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Studies of the Effects of Solvent Composition on the Association-Dissociation of Oxyhemerythrin as Determined by Analytical Gel Chromatography

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STUDIES OF THE EFFECTS OF SOLVENT COMPOSITION ON
THE ASSOCIATION-DISSOCIATION OF OXYHEMERYTHRIN
AS DETERMINED BY ANALYTICAL GEL CHROMATOGRAPHY

BY

KIM HOCK TAN

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF LOYOLA UNIVERSITY OF CHICAGO IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
AUGUST 1973

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ABSTRACT

Abstract of the dissertation entitled STUDIES OF THE EFFECTS OF SOLVENT COMPOSITION ON THE ASSOCIATION-DISSOCIATION OF OXYHEMERYTHRIN AS DETERMINED BY ANALYTICAL GEL CHROMATOGRAPHY submitted by Kim Hock Tan in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August 1973, Loyola University of Chicago.

The effects of the variations of pH, salt concentration and temperature on the association-dissociation equilibrium of oxyhemerythrin were investigated by means of frontal elution chromatography. From the properties of the elution profiles, the following conclusions concerning the rate and nature of the association-dissociation reaction of oxyhemerythrin were derived:

The rate and mechanism of the association-dissociation reaction varied in different pH regions. At an ionic strength of 0.01 the rate of equilibration is slow relative to rate of chromatography below pH 6.4, and rapid above pH 6.6.

Between pH 4.8 and 7.4 the order of the association-dissociation reaction (n) is 8, indicating that species of hemerythrin intermediate between monomer and octamer are not present in solution at any appreciable concentration. Between pH 8.0 and 8.8 the reaction can be best described as a consecutive dimerization of the type, monomer \rightleftharpoons dimer \rightleftharpoons tetramer \rightleftharpoons octamer. Above pH 9.0, the reaction becomes again

a rapid polymerization in which $n=3.7\pm0.4$. From variation in \bar{M}_w with protein concentration it is suggested that this is an octamer \rightleftharpoons dimer equilibrium in the range of protein concentration studied.

The pH dependence of the reaction indicates that at least one acidic group and one basic residue are present at each subunit contact. The acidic group has an apparent pK about 5 and an apparent heat of ionization of about 2.6 ± 1.6 Kcal/mole of monomer. This suggests a carboxyl group of an aspartyl or the glutymyl residue. The apparent pK of the basic residue was determined with less certainty. Its value appears to be between 9.0 and 9.5. These results are interpreted to mean that an $\epsilon\text{-NH}_3^+$ group of lysine and a carboxyl group of aspartate or glutamate participate in a salt linkage at each of the subunit contacts.

Several thermodynamic parameters were also measured for this reaction. The enthalpy change was found to be small and negative, the entropy change large and positive for the association equilibrium (Table XIX). It is apparent that the thermodynamic driving force is primarily entropic.

The effect of sodium sulfate on the association-dissociation reaction was also investigated. High salt concentration was found to promote dissociation. This is interpreted to mean that charge interaction plays an important role in maintaining the quaternary structure of hemerythrin. The thermodynamic data and salt data are also discussed in terms of domains of structured water at the contact regions of the subunits.

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To Dr. Nancy Egan, who helped to read the manuscript of this dissertation, I express my thanks and appreciation. To many of my fellow graduate students, I would like to express my sincere thanks for their invaluable friendship and help in the laboratory. To my wife Chio Hoon, whose love, encouragement and support are essential to the success of this work, I would express my greatest appreciation.

Finally, financial assistance from the Department of Biochemistry and Biophysics (1970-1973) is also gratefully acknowledged.

This dissertation is dedicated with all my love
to my parents, my brothers and sisters, my wife,
Chio Hoon and also my son, Timothy.

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CHAPTER I

INTRODUCTION

The Significance of Studying Macromolecular Interactions

The first quantitative studies of the quaternary structure of proteins were made by Svedberg and coworkers some forty years ago(Svedberg et al, 1929 and 1940). Since that work the subject of macromolecular interactions and subunit structure of proteins has gradually taken on an increased importance. Recent developments and discoveries in many areas of research, such as biochemical genetics, membrane chemistry and cellular regulation, has led to an increased awareness of the importance of macromolecular interactions in determining biological structure and function. A few examples may serve to illustrate this point. The first is provided by the interactions between regulatory subunits and catalytic subunits in aspartic transcarbamylase(Changeux et al, 1965). Other examples are the specific interactions between antigens and antibodies(Singer, 1965); the formation of such structural components as the viral coat and cellular membranes from smaller chemical constituents; and the complexing of E. coli repressor proteins with the lac operon DNA (Gilbert et al, 1966).

With some notable exceptions, proteins may be divided into two general categories. The first consists of proteins composed

of only a single polypeptide chain. The second consists of proteins composed of two or more polypeptide chains associated through non-covalent bondings. Among the few exceptions to these classifications are the γ -globulins, insulin, α -chymotrypsin etc. in which the several single chains are connected by disulfide bonds. The number of polymeric proteins and enzymes is far greater than those composed of a single chain (Klotz, 1967; Klotz, et al , 1970). Both classes of proteins can undergo a variety of interactions with small ions or molecules and also with other macromolecules. In non-polymeric proteins such as serum albumins, interactions with small ions, neutral molecules, etc. may result in changes in the conformation of the polypeptide chain (Steinhardt, 1969). On the other hand, in polymeric proteins, these interactions may often result in changes in quaternary structure superimposed over changes in tertiary structure. Reithel (1963) reviewed the association-dissociation behavior of many proteins and concluded that for many of them, biological activity is related to their degree of association. Change in the molecular weight of a protein may be followed with techniques commonly used for the measurement of certain

average properties of protein solution. Thus gel chromatography yields information about the weight average sieve coefficient while the weight average molecular weight is measured in a sedimentation experiment in the ultracentrifuge. Both parameters are a measure of the degree of polymerization of a protein in solution. Other transport methods such as electrophoresis and counter-current distribution have also been useful in studying interacting macromolecular systems (Nichol et al, 1964). Other methods that can be used which are not based on transport, are measurements of light scattering (Steiner, 1953); osmotic pressure (Guidotti et al, 1963; Guidotti, 1967) and polarization of fluorescence (Weber, 1953; Young and Potts, 1963). Among these various techniques, ultracentrifugation has been most frequently used for studying protein-protein interactions. In many cases studies of protein dissociation are carried out in the presence of high concentration of a third solute such as urea, guanidinium salts, detergents, etc.. These commonly used depolymerizing agents often force the equilibrium irreversibly to the monomeric species, and their use in dissociation studies is less desirable because the protein may also undergo denaturation. Dilution represents a mild method of promoting the dissociation of polymeric proteins. In order to obtain a sufficient degree of dissociation, one needs to use very low protein concentrations (e.g. $< 0.01\%$). Hence, the measurement of dissociation on

dilution becomes limited to those methods which allow measurement of molecular weights at very low protein concentrations. These concentrations are usually well below the normal operating range of conventional osmometers, light scattering instruments or ultracentrifuges. This difficulty is less serious for methods based on the use of the photoelectric scanning ultracentrifuge(Hanlon et al, 1962; Spragg et al, 1965) or on gel filtration(Winzor et al, 1963; Ackers et al, 1965). Winzor and Scheraga (1963) have experimentally demonstrated for the first time that all the theoretically predicted features of solute transport are observed in gel chromatography. Since then, the association-dissociation phenomenon of several polymeric proteins has been studied by gel filtration (Kakuichi et al, 1964; Winzor et al, 1964 and 1967; Ackers et al, 1965). Kellett (1967) has listed the major advantages of gel filtration over ultracentrifugation in a critical review of the study of interacting protein systems. Most important, the flexibility and simplicity of operation of gel filtration technique makes it the method of choice in the present investigation of the quaternary structure of hemerythrin.

Statement of Problem and Purpose

While the number of proteins known to be composed of subunits is large (Klotz, 1967), those for which a model

has been deduced describing their association-dissociation behavior is quite small (Klotz et al, 1970). Perhaps the best known and most extensively studied polymeric protein is hemoglobin. Many aspects of the chemistry and the three dimensional structure of hemoglobin have been elucidated (e.g. Antonini et al, 1970; Guidotti, 1967; Perutz et al, 1968). This information has provided important insights into the relationship between the structure and the function of this molecule. Hemerythrin, a nonheme-iron oxygen-carrying protein composed of identical subunits may be considered the counter-part of hemoglobin. Both proteins function as oxygen-carriers . However, much less is known about the association-dissociation behavior of hemerythrin. Nevertheless, hemerythrin affords several advantages in the study of subunit-subunit interactions. For one it is composed of identical subunits (Groskopf et al, 1966; Klippenstein et al, 1968), each with a distinct binding site for oxygen (Klotz, 1957), anions (keresztes-Nagy and Klotz, 1965) and sulfhydryl reagents(Keresztes-Nagy and Klotz , 1963). Furthermore, its macromolecular properties have also been well characterized (keresztes-Nagy,1962). Studies of the subunit interactions in hemerythrin can not only increase the knowledge about this protein but also may demonstrate the participation

of binding forces between subunits different from those already deduced for hemoglobin.

A Review of the Literature on Hemerythrin

The various physical and chemical properties of the nonheme-iron, oxygen-transport protein, hemerythrin, obtained from the marine worm Golfingia gouldii, have been the subject of intensive studies for the past fifteen years by Klotz and coworkers. The highest aggregate as well as the native form of hemerythrin is an octamer of molecular weight of 107,000 (Keresztes-Nagy et al, 1963), containing sixteen iron atoms, and capable of binding eight oxygen molecules (Klotz et al, 1955; Boeri et al, 1957; Keresztes-Nagy et al, 1963). The octameric protein can be dissociated into eight identical subunits of molecular weight of 13,500 by a numbers of reagents such urea and detergent(Keresztes-Nagy et al, 1963). Both hybridization experiments (Keresztes-Nagy et al , 1965) and sedimentation equilibrium studies(Klapper et al, 1968) have indicated that the octamers and monomers are in dynamic equilibrium. The hybridization experiments involved electrophoretic studies on succinylated and native hemerythrin. Succinylation results in molecules with two additional negative charges for each reacted lysine residues. If hybridization occurs between the succinylated and native

proteins, octamers with intermediate electrophoretic mobility should be found. Such hybridization has been demonstrated experimentally. Hemerythrin contains eight cysteine residues, one per monomer, which readily react with mercurials and other sulfhydryl modifying reagents (Keresztes-Nagy, 1962; Cress, 1972). Modification of the sulfhydryl groups causes a profound displacement of the association-dissociation equilibrium in favor of the monomer.

The earlier studies on hemerythrin by ultracentrifugation were mainly concerned with the effects of various iron-coordinating and non-iron-coordinating anions on the association-dissociation equilibrium of the protein at the neutral pH. The major conclusions drawn from this work may be summarized as follows: Various anions such as azide, thiocyanate, chloride etc. were found to coordinate the iron atoms of hemerythrin. Coordination of these ligands with iron resulted in ligand-dependent changes in the absorption spectra of hemerythrin (Keresztes-Nagy et al, 1965), as well as to changes in the reactivity of the cysteine side chains and the position of the apparent association-dissociation equilibrium between octamer and monomer. In contrast to the above mentioned anions, perchlorate and nitrate ions were found not to bind to iron(Garbett et al, 1971a). The binding of these anions at non-iron sites on hemerythrin resulted in decreased dissociation of octameric hemerythrin.

Only recently have techniques other than ultracentrifugation been used to study subunit-subunit interactions in hemerythrin. For example, Langerman and Sturtevant (1971) used the microcalorimetric method and Rao and Keresztes-Nagy (1972b) applied the technique of analytical gel chromatography. The results of Rao et al and Langerman et al were in general agreement with that obtained from ultracentrifugation studies. In addition, the work of Langerman et al indicated that an amino acid residue with a pK about 8 was involved at the subunit contacts.

The early studies on hemerythrin were carried out almost exclusively in the neutral pH region and with various ligand-bound forms of met-hemerythrin. As a consequence there is very little information concerning the effects of acids, bases and salts etc. on the degree of dissociation or on the nature of the subunit interactions in hemerythrin. Recently, Langerman et al (1971) using microcalorimetry, studied the association-dissociation equilibrium and ligand-binding properties of hemerythrin between pH 5.6 and 9.2 in the 10^{-4} M range of protein concentrations (Moles of monomers/ liter). These authors assumed that the reaction was octamer \rightleftharpoons monomer equilibrium between pH 5.6 and 9.2. The equilibrium constant and the related thermodynamic parameters were then derived, and were found to be in fair agreement with the results from sedimentation experiments. However, the use of microcalorimetry in investigating

hemerythrin has certain draw backs. This method does not provide information about the rate and mechanism of the association-dissociation process. Thus, any pH or temperature dependent changes in rate or mechanism may be overlooked. This may be illustrated by the example of hemoglobin. Field and O'Brien (1955) observed that the tetrameric hemoglobin dissociated into dimers as the pH was decreased below pH 6.0 and then into monomer on further decreasing the pH.

Contradictory reports also exist in the literature on the rate of the association-dissociation reaction of hemerythrin. For example, in sedimentation velocity studies, Klapper et al (1966) observed that the dissociation equilibrium for met-Hr-SCN complex at pH 6.8 was attained only slowly. Rao et al (1972b) using gel filtration to study the dissociation of the same form of hemerythrin under similar conditions found that the rate of equilibration between octamer and monomer was relatively rapid. The explanation for the differences reported by these two groups of workers may be related to "high pressure effects" generated during the high speed sedimentation velocity experiments. (Joseph and Harrington 1966; Bethune et al, 1967; Penniston, 1971) or to some other factors. The disparity of results could also be explained by the assumption that the two sets of experimental conditions were similar but not identical and that the

equilibration rate of the hemerythrin system is sensitive to small changes in pH, ionic strength and temperature etc..This latter assumption will be examined in this study.

Aims and Objectives

Despite the great progress in the understanding of hemerythrin structure and function, there still remain a number of formidable challenges. One important aspect of the behavior of this protein which has not received much attention from previous work is: how the effect of enviromental conditions (i.e. pH, ionic strength and temperature etc.) control the extent and mode of association-dissociation; what forces hold the subunits together. The present investigation of the association-dissociation of hemerythrin was therefore undertaken with these basic aims in mind. The first object is to reexamine the rate and stoichiometry of the reaction. The earlier workers reported that hemerythrin, in the concentration range of 10^{-5} M and above, tends to form octamers so strongly that no significant concentrations of species intermediate between octamer and monomer can be detected (Klapper et al, 1968; Klotz et al, 1970). In addition, they assumed that the nature (rate and stoichiometry) of the association-dissociation reaction of hemerythrin was not appreciably affected by the pH (Langerman et al, 1971). This situation might well be an

oversimplification, because in these studies only one reaction mechanism, octamer \rightleftharpoons monomer equilibrium was assumed and used to account for the data. Moreover, the study of the effect of pH was not carried out in any great detail. Therefore, the rate and the stoichiometry of the association-dissociation reaction of oxyhemerythrin will be examined over a wide range of pH, ionic strength and temperature.

A second objective is to obtain information about the forces stabilizing the quaternary structure of hemerythrin. The nature of molecular forces is universal and our understanding of such forces derived from studies of small molecules, particularly those which have the same groupings of atoms as the side groups and the backbone of protein's, promotes our understanding of the forces within and between protein molecules. There are various types of forces which may be involved in the subunit-subunit interactions and contribute to a varying degree to the maintenance of the quaternary structure of a protein molecule. These forces include electrostatic interactions, hydrophobic interactions, hydrogen bondings, and so on. Though it is conceivable that all of these forces may participate, the relative contribution each makes to the stability of the subunit structure varies from protein to protein.

The third objective is to attempt to identify some of the amino acid residues participating in the subunit contact

regions. This is possible for ionizable amino acid side chains if reliable values of pK and apparent heats of ionization can be obtained. Chemical modification study may also permit identification of specific amino acid residues at subunit contacts.

Finally, thermodynamic parameters will be derived from the effects of pH, temperature and ionic composition on the association-dissociation equilibrium of hemerythrin. The analysis of the thermodynamic data should give us some insight about the driving force of the reaction and also the possible role of solvent in the formation of quaternary structure of hemerythrin.

The Study of Protein-Protein Interactions by Gel Filtration

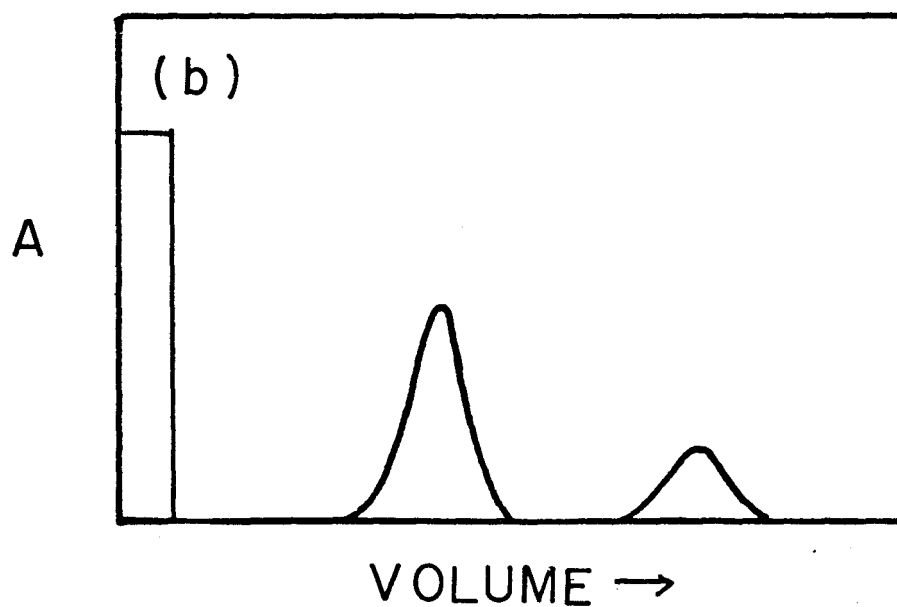
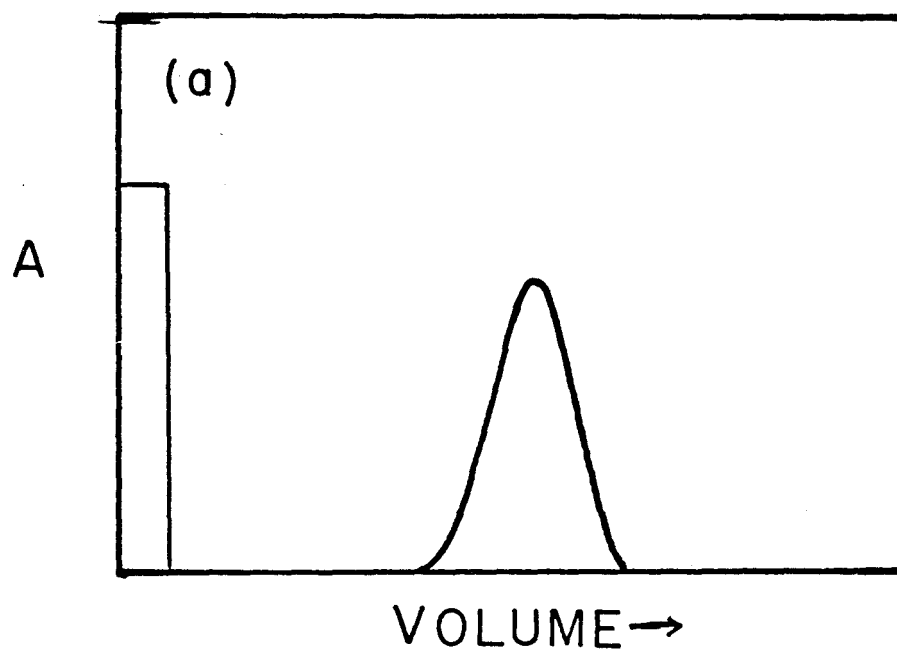
In general there are two ways to detect and study interacting macromolecular systems by molecular-sieve chromatography. These methods differ in the volume of solute applied to the column and are classified as zonal and frontal chromatography. Kellett(1967) has reviewed the usefulness of these two procedures for studies of protein-protein interactions. The following paragraphs will briefly describe and compare the two approaches. I shall especially point out their current status as applied to the study of macromolecular interactions.

In zonal chromatography, a small volume of the sample (solute) is applied to the column. The solute then

migrates through the column in a manner described by a Gaussian distribution. The elution volume of the sample corresponds to the volume at which the concentration of the sample is a maximum in the elution profile (Figure 1). Zonal procedure is often used for fractionation and purification work but it has limited application in the studies on protein-protein interactions. For slow reactions (when the rate of attainment of equilibration between the interacting species is slow compared with their rate of separation by differential transport), zonal analysis provides a simple means of determining an equilibrium constant. Because the interacting species behave as though they were noninteracting entities, they can be resolved into separate peaks. The areas under these peaks afford a direct measure of the equilibrium concentrations of the various species in the initial solution.

For a rapid self-associating (or dissociating) system, zonal analysis is not suitable. As the zone of solute moves down the column, it becomes progressively more dilute because of eddy diffusion around the gel particles of bed material. As the protein becomes more dilute the weight average molecular weight and therefore the rate of migration continually decreases. It is difficult to define the weight average concentration to which the final elution volume of the peak refers. Very recently, Zimmerman and Ackers (1971) have investigated

Figure 1. Diagrammatic representation of zonal chromatography. (a) the single moving zone of a homogeneous substance; (b) separation of a mixture of two substances with different size.



reversible self-associating solutes by computer simulation. They found that when such systems are studied by zonal analysis, completely misleading information is obtained regarding the equilibrium constant.

In frontal analysis a sufficient large volume of solute solution is applied to the gel column to produce an elution profile containing a leading sharp boundary, a plateau of constant protein concentration and a trailing boundary. The elution volume of the leading boundary is then defined as the volume corresponding to the equivalent sharp boundary (first moment of the leading boundary of the elution profile as described by Longsworth, 1943), or to the centroid elution volume (Ackers and Thompson, 1965). That is, the volume at which the boundary would have eluted had it remained perfectly sharp in the absence of diffusion. This elution volume (centroid volume or equivalent sharp boundary) refers specifically to the concentration of protein in the plateau regions. Information about the rate of reaction, stoichiometry and equilibrium constant may be derived from the analysis of both the leading and trailing boundaries of the elution profile. This is applicable to the fast as well as to the slow reactions. In the latter case the interacting species can be separated completely in which case they appear as individual boundaries. The concentration of each subspecies is proportional to its plateau

height, which when multiplied by the total initial solute concentration, C_0 , will give its concentration in the reaction mixture.

Theoretical Treatment of Frontal Chromatography

1. General Transport Theory Developed for Ultracentrifuge and Electrophoresis by Gilbert

In 1955 Gilbert developed a theory of mass transport which describes the behavior of a protein undergoing a rapid association-dissociation reaction in either a centrifugal or electrical field. For the simple reaction,



the equation which describes the transport process is

$$(1) \quad C_m + C_p = \left((1/nK') (\delta / 1 - \delta) \right)^{1/n - 1} \left[(1 + 1/n \cdot \delta / 1 - \delta) \right]$$

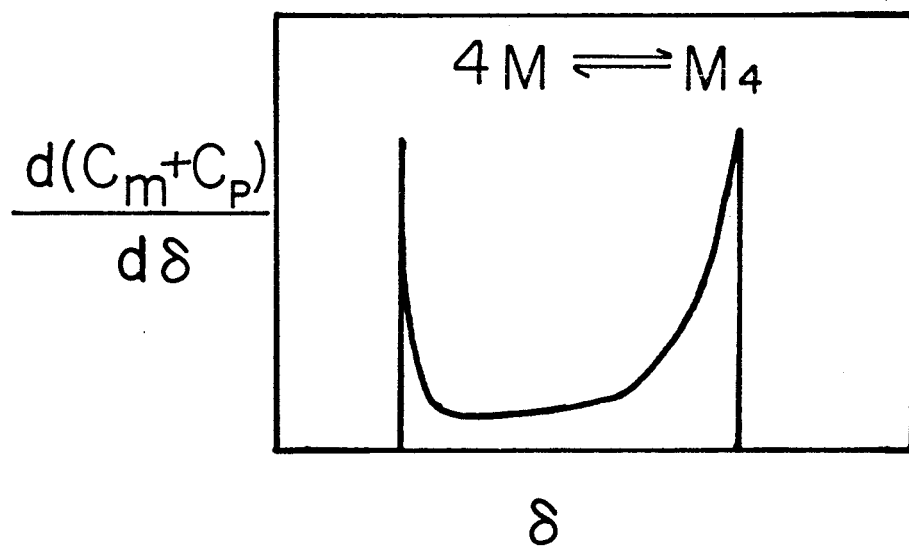
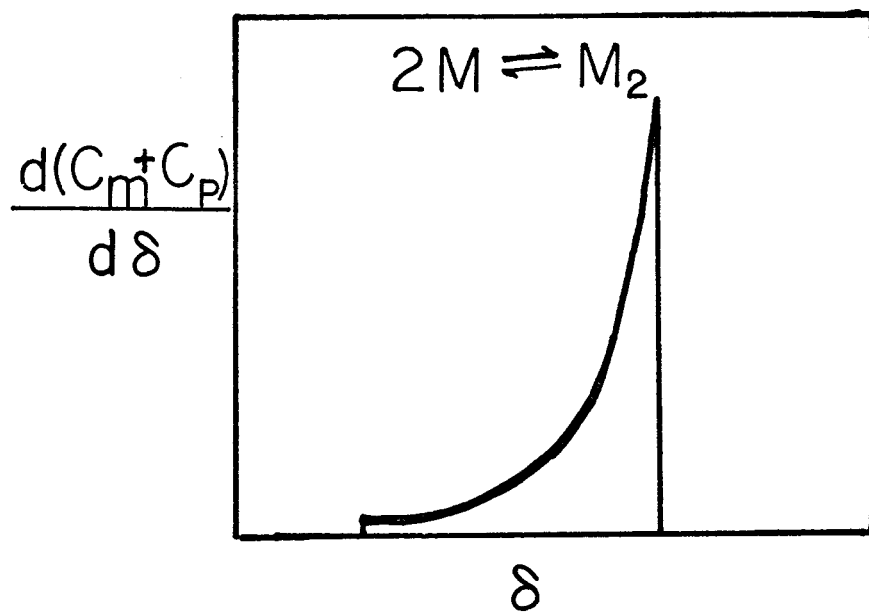
In this equation, $K' = C_p / C_m^n$, $\delta = \frac{\eta - v_m}{v_p - v_m}$ and $\eta = x/t$.

v_m and v_p are the sedimentation velocities of monomer and polymer, and x is the distance of the macromolecule from the axis of rotation in the ultracentrifuge cell. Since the sedimentation pattern is usually recorded as concentration gradient versus position, it is convenient to differentiate Eq (1) with respect to δ , thereby one obtains Eq (2),

$$(2) \quad \frac{d(C_m + C_p)}{d\delta} = \frac{1}{(n-1)} \left(\frac{1}{nK'} \right)^{1/n-1} \delta^{\frac{(2-n)}{(n-1)}} \left(\frac{1}{1-\delta} \right)^{\frac{2n-1}{n-1}}$$

In his derivation of the transport equations, Gilbert made the assumption that there is an instantaneous reestablishment of equilibrium during differential transport of monomer and polymer. In this event, monomer and polymer must always coexist at all positions in the centrifuge cell. It does not necessarily follow, however, that the sedimenting boundary will be unimodal. As Gilbert (1959) has pointed out, partial resolution of a reaction boundary into components may occur even for a rapidly interacting system. One can demonstrate this by taking the second derivative of Eq (1) with respect to δ and equating it to zero. When this is done, a single minimum is found at $\delta = (n-2)/3(n-1)$. This result expresses the most dramatic prediction of the Gilbert theory. Whereas only a single sedimenting peak will be observed for dimerization ($n = 2$), for higher order polymerization reaction ($n \geq 3$) the reaction boundary may resolve into two components even though reequilibration is instantaneous. This important prediction is depicted in Figure 2a and b, which contrasts theoretical sedimentation velocity patterns for dimerizing and tetramerizing systems. It must be emphasized that neither of the components in the latter pattern can be identified with a distinct macromolecular entity, since both monomer and polymer

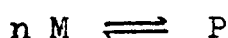
Figures 2a and b. Comparison of theoretical sedimentation velocity patterns for dimerizing and tetramerizing systems, $n M \rightleftharpoons P$. Sedimentation is from left to right (Gilbert, 1959).



coexist in equilibrium at every position where the concentration is finite.

2. Application of Transport Theory to Gel Filtration by Ackers and Thompson

Although Gilbert originally formulated his theory with sedimentation and electrophoresis in mind, he recognized the analogy between these and other methods of mass transport such as chromatography. The analogous theory was subsequently elaborated by Ackers and Thompson (1965) for molecular-sieve chromatography (gel filtration). Ackers and Thompson formulated the following expression relating macromolecular concentration to position in the trailing boundary of the elution profile for a rapidly associating-dissociating system of the type,



$$C_m + C_p = (1/nK') (\phi/1 - \phi) \frac{1}{n - 1} (1 + 1/n \cdot \phi/1 - \phi) \quad (3)$$

In this equation

$$\phi = \frac{\sigma_m - v'}{\sigma_m - \sigma_p} \quad (4)$$

and

$$v' = \frac{V' - V_0 - S}{V_i} \quad (5)$$

v' is the elution volume at any position on the trailing boundary, s is the volume of solute applied to the column, V_0 is the void volume, and V_i is the internal volume of the column. Equation (3) is identical in form to Gilbert's equation [Eq (1)] for sedimentation and electrophoresis. Differentiation of Eq (3) with respect to v' gives, for the gradient of total solute,

$$(6) \quad \frac{d(C_m + C_p)}{dv'} = \frac{1}{(n-1)} \cdot \frac{1}{(\sigma_m - \sigma_p)} \left(\frac{1}{nK'} \right)^{\frac{1}{n-1}} \phi^{\frac{2-n}{n-1}} \left(\frac{1}{1-\phi} \right)^{\frac{2n-1}{n-1}}$$

Thus, a unimodal gradient curve is predicted for dimerization ($n=2$) and a bimodal one for higher order polymerization ($n \geq 3$). The minimum in the bimodal gradient for $n \geq 3$ occurs at the effluent volume

$$V'_{\min} = V_0 + S + \left[\frac{2n-1}{3(n-1)} \cdot \sigma_m + \frac{n-2}{3(n-1)} \cdot \sigma_p \right] V_i \quad (7)$$

With the aid of Eq (5) we can show that $V'_{\min} = v'_m \cdot V_i + V_0 + S$. Substituting this expression for V'_{\min} into Eq (7) and rearranging yields

$$n = \frac{3v'_m - \sigma_m - 2\sigma_p}{3v'_m - 2\sigma_m - \sigma_p} \quad (8)$$

Equation (8) can be used to estimate n . Finally, Eq (3) and (6) predict virtually the same concentration dependence of gradient shape and elution rates as does Gilbert theory for the analogous quantities in sedimentation and electrophoresis. It should be pointed out that the transport equation (Eq (3)) derived by Gilbert was obtained under the assumption of negligible diffusion and thus Eq (3) represents an ideal system. Similarly, Ackers et al made the assumption that the axial dispersion effect is negligible in their derivation of the transport for gel chromatography.

3. Cann's Transport Theory for Ligand-Mediated Interactions

Cann and Goad (1970a and 1970b) have extended the Gilbert theory to include association-dissociation reactions that are ligand mediated. An example of a ligand-mediated reaction is



in which a macromolecule, M , associates into a polymer containing n monomeric units, through the mediation of m small molecules or ions X . The set of conservation equations for sedimentation in a sector-shape cell have been derived. These equations have been solved numerically for both the dimerization and tetramerization reactions in which the rates of reaction are very much larger than the rates of diffusion and sedimentation (1970b). In certain

respects, the solution for the dimerization reaction is more revealing with regard to the major differences between the Cann's theory and the Gilbert theory. While the Gilbert theory predicts that the sedimentation pattern of the system $\text{monomer} \rightleftharpoons \text{dimer}$ will show a single peak for instantaneous equilibration, Cann's theory indicates ligand-mediated dimerization may show two peaks due to the production of concentration gradients of the unbound ligand in the ultracentrifuge cell at high centrifugal fields. Indeed, Cann's theory has been experimentally demonstrated by Weisenberg and Timasheff (1970) in their study of the vinblastine mediated dimerization of calf brain microtubule subunit protein, tubulin. Also Morimoto and Kegeles (1971) studied the proton and calcium ion mediated association of lobster hemocyanin. In both cases, the sedimentation patterns showed bimodality in spite of the fact that the reactions were dimerization.

4. Relationship Between Cann and Gilbert Theory

Under some specific experimental conditions, Cann's theory may become indistinguishable from Gilbert's theory. Thus, when the ligand concentration is much greater than protein concentration, the amount of bound ligand sedimenting with the protein will not be sufficient to allow the establishment of a ligand concentration gradient in the ultracentrifuge cell. Similarly, if the centrifugal field is small such that the rate of sedimentation of the protein is slower than the rate of

diffusion of the ligand then a ligand concentration gradient will not be established in the centrifuge cell. Under these conditions, the system effectively approaches the case of simple dimerization considered by Gilbert. Finally, when the rate of equilibration between the monomer and polymer is slow in comparison with the time of the sedimentation experiment, the components of the sedimentation pattern corresponding to monomer and dimer (or polymer) are well separated from one another. In that event, the areas and sedimentation coefficients of the components in the sedimentation pattern can be interpreted as though they are noninteracting species.

Qualitative Solution of Transport Equations: Gel Chromatography

1. Relationship Between Rate of Association-Dissociation and Shape of the Elution Profile

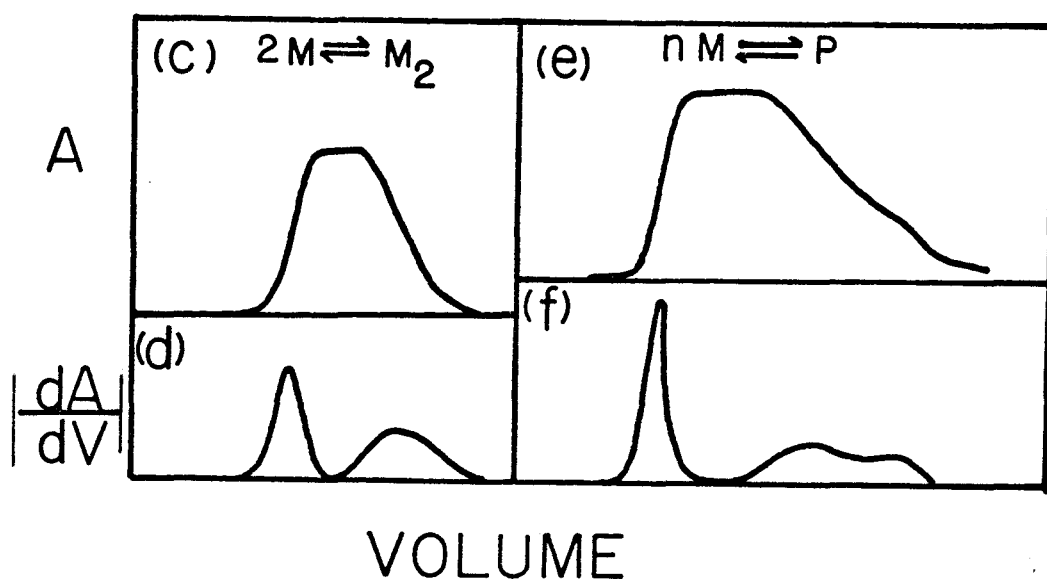
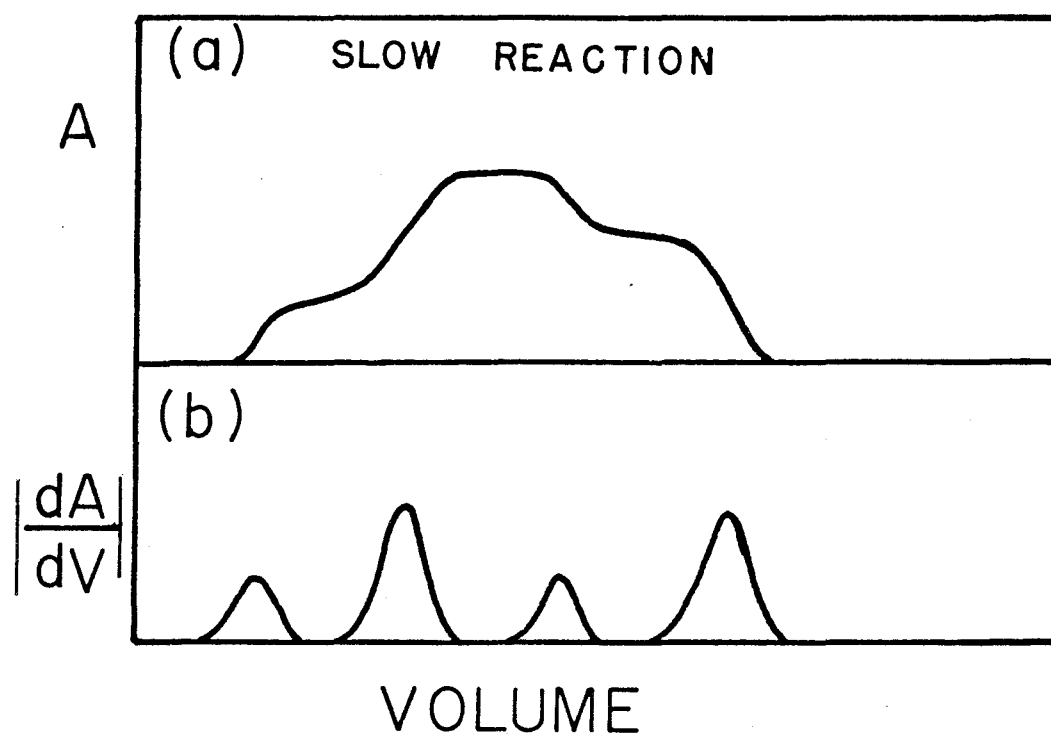
The gel filtration experiments on reversibly associating-dissociating macromolecules present no interpretative difficulties if the rates of reaction are sufficiently slow compared to the time required for separation of the various species. Accordingly, the elution profiles will resolve into two or more boundaries corresponding to reactants and products. This will be observed both for the ascending and descending boundaries of the elution diagram. The integral and differential elution patterns for a slow reaction $n M \rightleftharpoons M_n$ are shown in Figures 3a and b.

For the rapid reequilibration between species during their differential transport, the integral elution profile will exhibit a single sharp leading boundary, a plateau region, and a trailing boundary, as shown in Figures 3c and d for a dimerization reaction. The properties of the trailing boundary is dependent on the stoichiometry of the reaction in question in accord with the Gilbert theory.

2. Relationship Between Stoichiometry of the Rapid Association-Dissociation Reaction and Behavior of The Trailing Boundary

For simple reaction of the type, $n M \rightleftharpoons M_n$, the Ackers and Thompson theory predicts a unimodal trailing boundary for dimerization ($n=2$) and an unresolved bimodal trailing boundary when $n \geq 3$. Figures 3c and d represent a rapid dimerization reaction while Figures 3e and f depict a rapid association-dissociation reaction for which $n \geq 3$. The properties of the trailing boundary for a ligand-mediated reaction, $nM + mX \rightleftharpoons M_nX_m$, is more complicated since they vary with the experimental conditions and the cooperativeness of the interaction itself. In that case, a ligand-mediated dimerization reaction may show bimodality as would be predicted by Gilbert theory for a simple polymerization ($n \geq 3$). In general, the shape of the elution profiles predicted by Cann's theory can be determined only by numerical solution of the relevant set of exact conservation equations.

Figure 3. Integral and differential elution profiles of frontal experiments. (a) and (b) : slow association-dissociation reaction; (c) and (d) : rapid dimerization; (e) and (f) : rapid polymerization.



CHAPTER II

MATERIALS AND METHODS

Materials

Oxygenated hemerythrin, isolated from the coelomic fluid of marine worm, Golfingia gouldii, was used throughout the course of this investigation. All chemicals used in the experiments were reagent grade, were obtained from commercial sources, and were not subjected to further purification. Marker proteins used in the calibration of Sephadex columns are listed as follows: bovine serum albumin, ovalumin, and horse myoglobin were obtained from Nutritional Biochemical Corporation; chymotrypsinogen from Schwarz Bio-research, Inc.; and cytochrome c from Sigma Chemical Co. Blue dextran and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Inc.. Live Golfingia gouldii were supplied by the Marine Biological Laboratories, Woods Hole, Massachusetts.

Isolation and Purification of Oxyhemerythrin

Hemerythrin was isolated from the coelomic fluid of the sipunculid worm, Golfingia gouldii by the procedure of Klotz et al (1957). The coelomic fluid of the worms were collected and the red cells sedimented by centrifugation. The cells were then washed several times with 2.5 % saline after which they were lysed in cold distilled water. The cellular debris was removed by centrifugation at low speed and the supernatant dialyzed against 20% ethanol (v/v) . During dialysis red

crystals of oxyhemerythrin formed. The crystals were collected and washed with 20% ethanol. A white layer formed on top of the crystals. This layer was removed by suction resulting in some loss of protein. The crystals were then stored wet at 3 °C. Precautions were taken to prevent the crystals from becoming dry as drying results in denaturation (Keresztes-Nagy, 1962).

Preparation of Protein Solutions

Stock Solutions of oxyhemerythrin were prepared by dissolving an amount of crystalline material sufficient for one week into 5.0 ml of 0.01 M Tris-cacodylate buffer, pH 7.0 and 5°. The solution was centrifuged for 20 minutes at 3,000xg and 5°. The supernatant solution was collected and the concentration of protein determined spectrophotometrically. Sample protein solutions of given concentrations were prepared by adding a measured amount of stock solution to a volumetric flask and diluting with the appropriate buffer. The protein concentration was again determined by measuring absorbancy at 280 nm. A specific absorbance of 2.73 mg/ml/cm was used to calculate protein concentration (Keresztes-Nagy and Klotz, 1965).

Preparation of Buffer Solutions

Buffer solutions of an ionic strength of 0.01 were prepared by dissolving Tris and cacodylic acid in deionized water. Buffers of higher ionic strengths were prepared by addition of an appropriate amount of solid sodium sulfate to 0.01 M Tris-cac buffer. The pH was measured with a Corning research pH meter. The pH of

the standard buffers used to calibrate the pH meter was always within 1.5 units of the buffer solutions being measured. Both the standard buffers and the buffer solutions were kept in the same temperature bath prior to pH measurement. After each buffer solution was prepared it was filtered through a 0.22 μ Millipore membrane and partially degassed in order to minimize the formation of bubbles within the gel columns.

Preparation and Equilibration of Sephadex Columns

Chromatography columns consisted of 1.5x60 cm glass cylinders sealed at the ends with inlet and outlet plungers supplied by Chromatronix. Temperature regulation was achieved with the aid of Phoenix glass jackets surrounding the columns. Each column was packed with Sephadex G-75 gel which had been swollen for three days in 0.01 M Tris-cac buffer, pH 7.0. Columns were usually packed to a height of about 51 cm by application of a partially degassed slurry of Sephadex G-75 from which the fine had been carefully removed. Prior to each experiment, columns were equilibrated for 24 hours with the appropriate buffer solution. A constant flow rate was maintained for the entire period the column was in use in order to preserve the column's characteristics throughout a series of experiments.

Application and Elution of Protein Samples

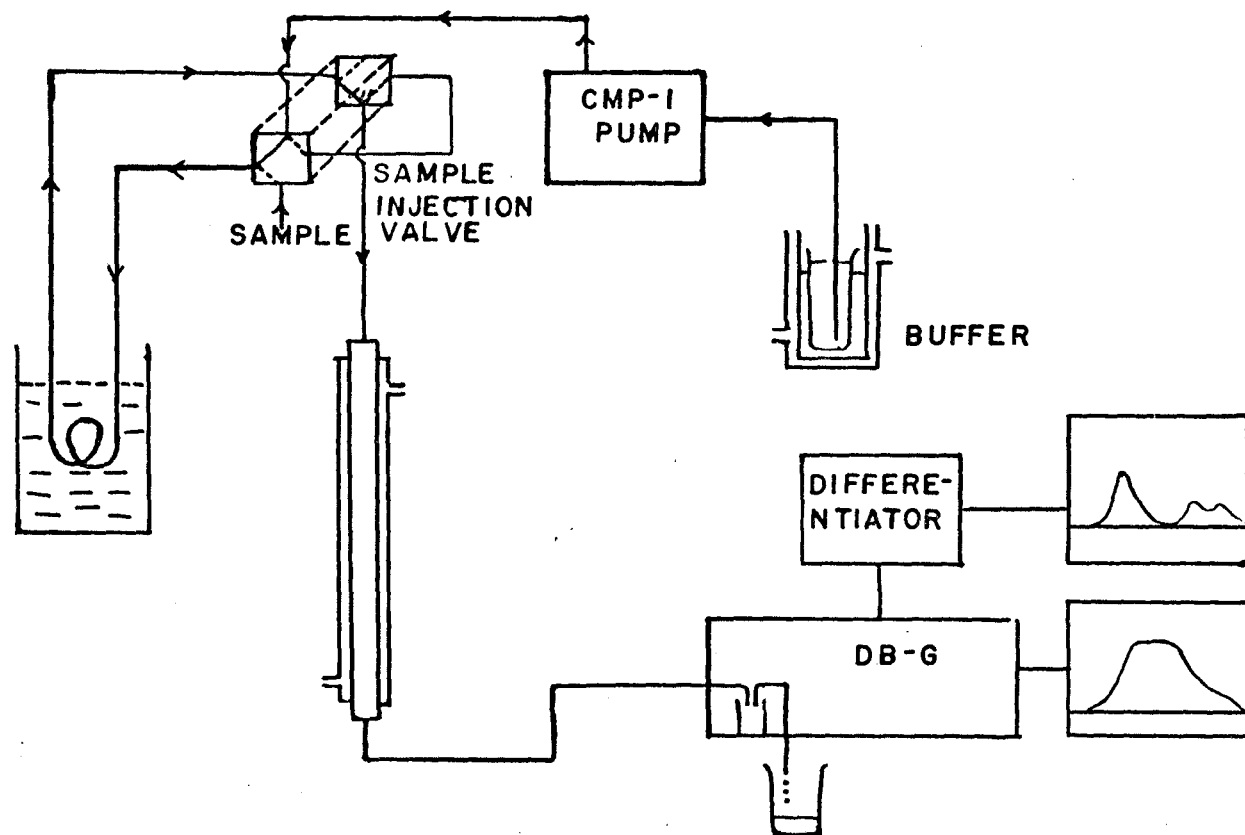
Chromatography experiments were carried out in the following manner. The inlet tube of a well-packed and equilibrated Sephadex column was connected to a buffer reservoir and a sample loop by means of a three-way valve. The sample solution and

buffer were alternately applied to the column with a Chromatronix pump operating on compressed air at a pressure of 80 psi. The volume of sample applied was 28-36 ml for frontal chromatography. The outlet plunger was connected to a Beckman flow cell (0.3 ml capacity and 1.0 cm light path) by teflon tubing. The effluent was monitored with a Beckman DB-G spectrophotometer. The wavelength at which the sample was monitored was set such that the absorbance measured at the peak of the elution profile (or the plateau of the elution profile) was between 0.1 and 1.0. The elution profile was recorded with a Beckman 10-inch recorder. The flow rate was set at 24 ml/hr. The effluent was periodically collected in a 10-ml burette thereby providing a check on the flow rate. The flow rate was found to be essentially constant. Because the three-way valve on the inlet tube allowed sample application to occur without interruption of the flow, it was possible to determine the elution volume directly from the recorded elution profile with an accuracy of 1 to 2%. The first differential of the elution profile was also recorded with the aid of a "curve differentiator" and a Photovolt Varicord model 43 recorder. Thus for every experiment both the integral and differential elution profiles were displayed simultaneously. The schematic of the monitoring system and the circuit diagram of the "curve differentiator" are shown in Figures 4 and 5, respectively.

Calibration of Sephadex Columns

There exists a relationship between the sieve coefficients

Figure 4. Schematic of the experimental setup and the monitoring system for the Sephadex column.



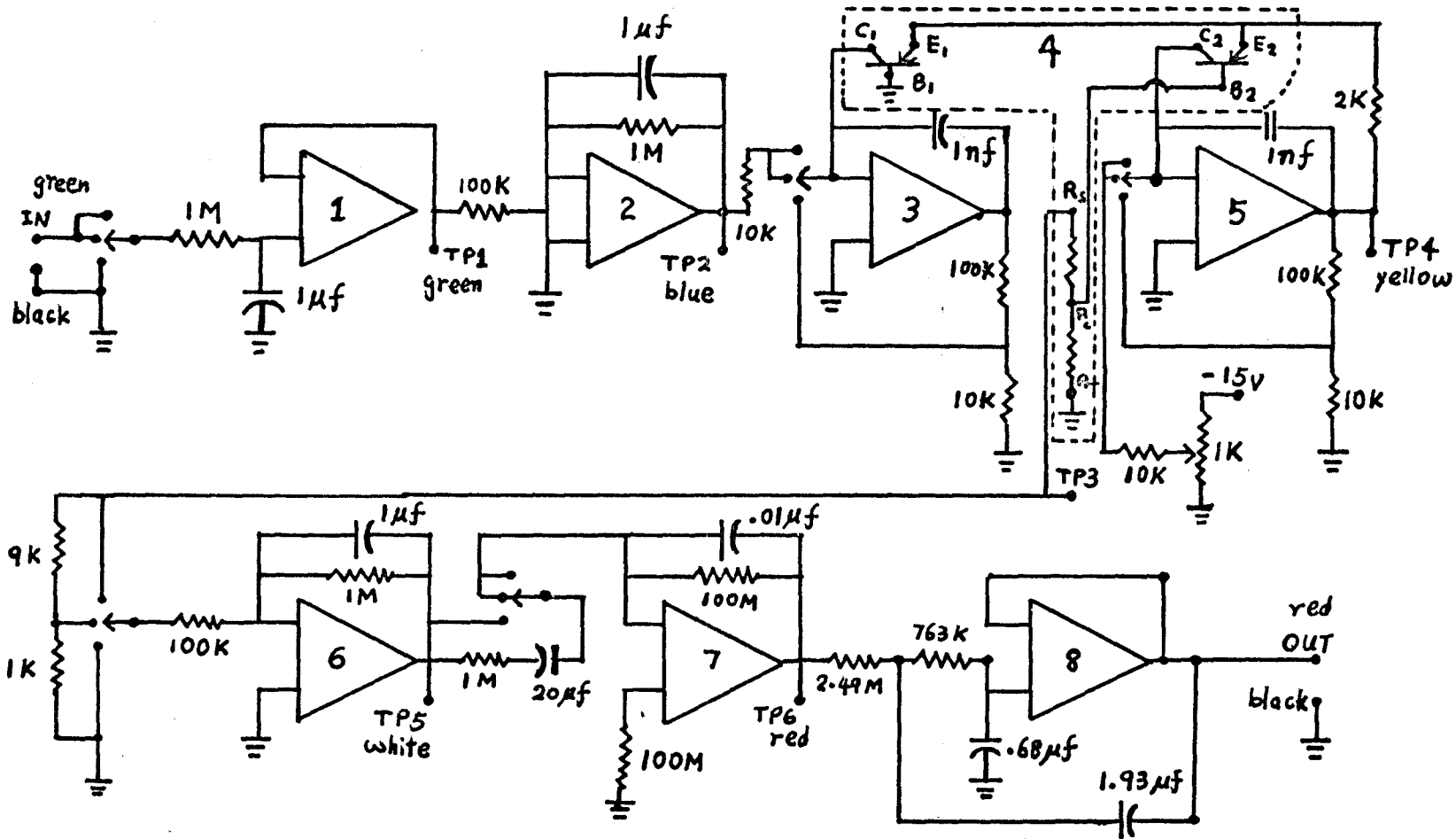
SCHEMATIC OF THE MONITORING SETUP OF SEPHADEX COLUMN

Figure 5. Circuitry diagram of differentiator.

Explanation : 1,2,6 and 8 : General purpose operational amplifiers.

3,5 and 7 : FET multiplication operational amplifiers.

4 : Logarithmic module (matched transistors).



(or partition coefficients) of a series of proteins and their respective molecular weights. This relationship, which is only valid over a relatively narrow range of molecular weights, is expressed by Eq.(9),

$$\sigma = -A \log M + B \quad (9)$$

Equation (10) defines the sieve coefficient, σ ,

$$\sigma = (V_e - V_o) / V_i = (\bar{V} - V_o) / V_i \quad (10)$$

In Eq.(10), V_e and \bar{V} are the elution volume and the centroid elution volume, respectively; V_o represents the void volume; and V_i , the internal volume is the difference between the total volume, V_t , and the void volume. In Eq.(9), A and B are column calibration constants and were determined experimentally. This was accomplished by applying 0.3 ml of a solution of a protein of known molecular weight to a Sephadex column. The elution volume of the standard was then determined from the position of the peak in the effluent profile (Figure 6). The sieve coefficient was then calculated according to Eq.(10) from a knowledge of V_o and V_i . The procedure was repeated for a number of different protein standards. The void volume, V_o , was defined as the elution volume of a marker substance that is totally excluded from the gel matrix. This marker used was blue dextran. The total volume, V_t , was the elution volume of a marker substance that is totally included within the gel matrix. The marker used was potassium chromate. The internal volume, V_i , is then equal to $(V_t - V_o)$. The sieve coefficients along with the molecular weights of the various protein standards are collected in Table I.

Figure 6. Schematic elution diagrams illustrate three types of solute behavior: (a) total exclusion; (b) a penetrant molecule; (c) a small totally nonexcluded molecule.

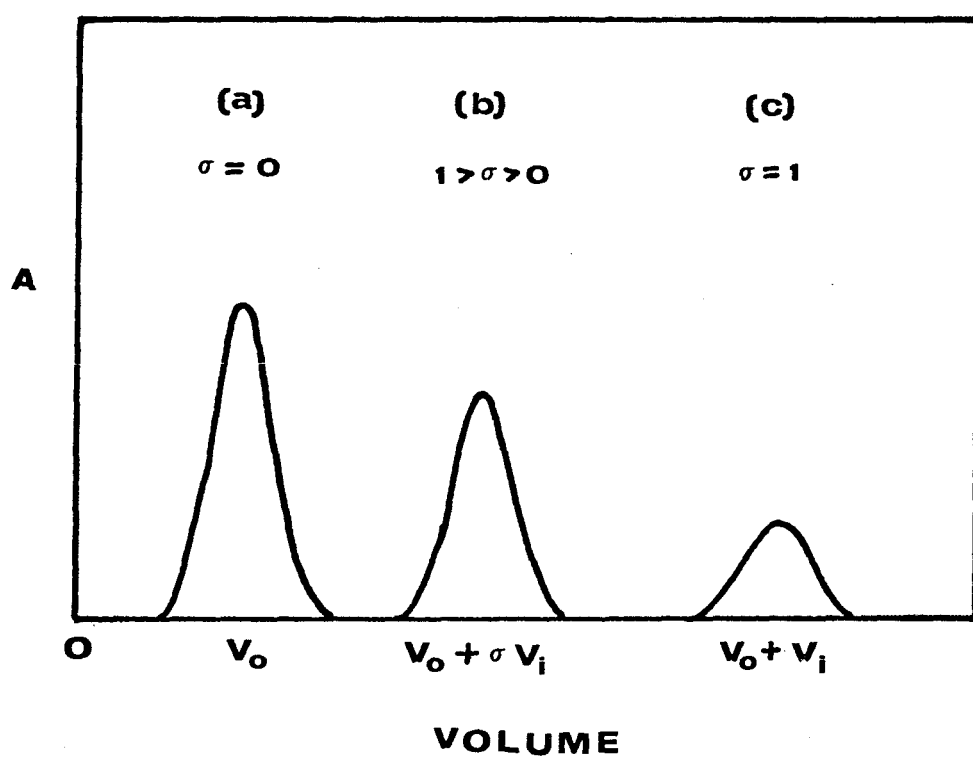


TABLE I
Calibration data of Sephadex G-75 column at 25°

Marker	M.W.	-log M.W.	V _e (ml)	σ	σ [#]
Blue dextran	-----	-----	23.52	0.000	---
BSA	67,000*	-4.8261	26.53	0.066	---
OVA	45,000*	-4.6532	30.37	0.150	0.160
CGN	25,000*	-4.3979	38.23	0.322	----
Trypsin	24,000*	-4.3802	-----	-----	0.355
Mb	17,000*	-4.2304	42.25	0.410	0.415
Cyt c	13,000*	-4.0934	45.45	0.480	0.475
Hr	13,500**	-4.1303	45.67	0.485	----
K ₂ CrO ₄	-----	-----	69.20	1.000	----

* Values are from Determan, p.108(1969)

** Hemerythrin monomer(M.W. 13,500) is readily obtained at pH below 5.2 when protein concentration is less than 20 μg/ml.

Values are obtained from Henn and Ackers(1968).

The data in Table I was then plotted and the best straight line through the points was obtained by the method of least squares with the aid of an Olivetti Underwood Programma 101 computer. A and B were determined from the slope and intercept of the calibration curve (Figure 7). With the aid of Eq.(9), an experimentally determined value of σ could be converted directly to M (or \bar{M}_w for an interacting system). Alternately, the calibration curve could be used directly to yield values of M from the value of σ .

Sieve Coefficients of Various Species of Hemerythrin

The sieve coefficient for monomeric hemerythrin, σ_1 , may be obtained in two ways. The first method consisted of measuring the weight average sieve coefficient, $\bar{\sigma}_w$, as a function of protein concentration. Extrapolation to zero protein concentration yielded the sieve coefficient of monomer. This procedure has been adopted by several works to determine the monomeric sieve coefficient when the pure monomer could not be readily obtained (Henn and Ackers, 1968). Using this procedure, σ_1 , was found to be 0.489. This is illustrated in Figure 8 and Table II.

TABLE II

Concentration Dependence of $\bar{\sigma}_w$ in 0.01 M
Tris-cacodylate Buffer at pH 7.00 and 25°

C_o ($\mu\text{g/ml}$)	30.0	20.9	14.0	10.0	5.0	2.0
$\bar{\sigma}_w$	0.078	0.087	0.109	0.128	0.230	0.354

Figure 7. Calibration plot of Sephadex G-75 column.

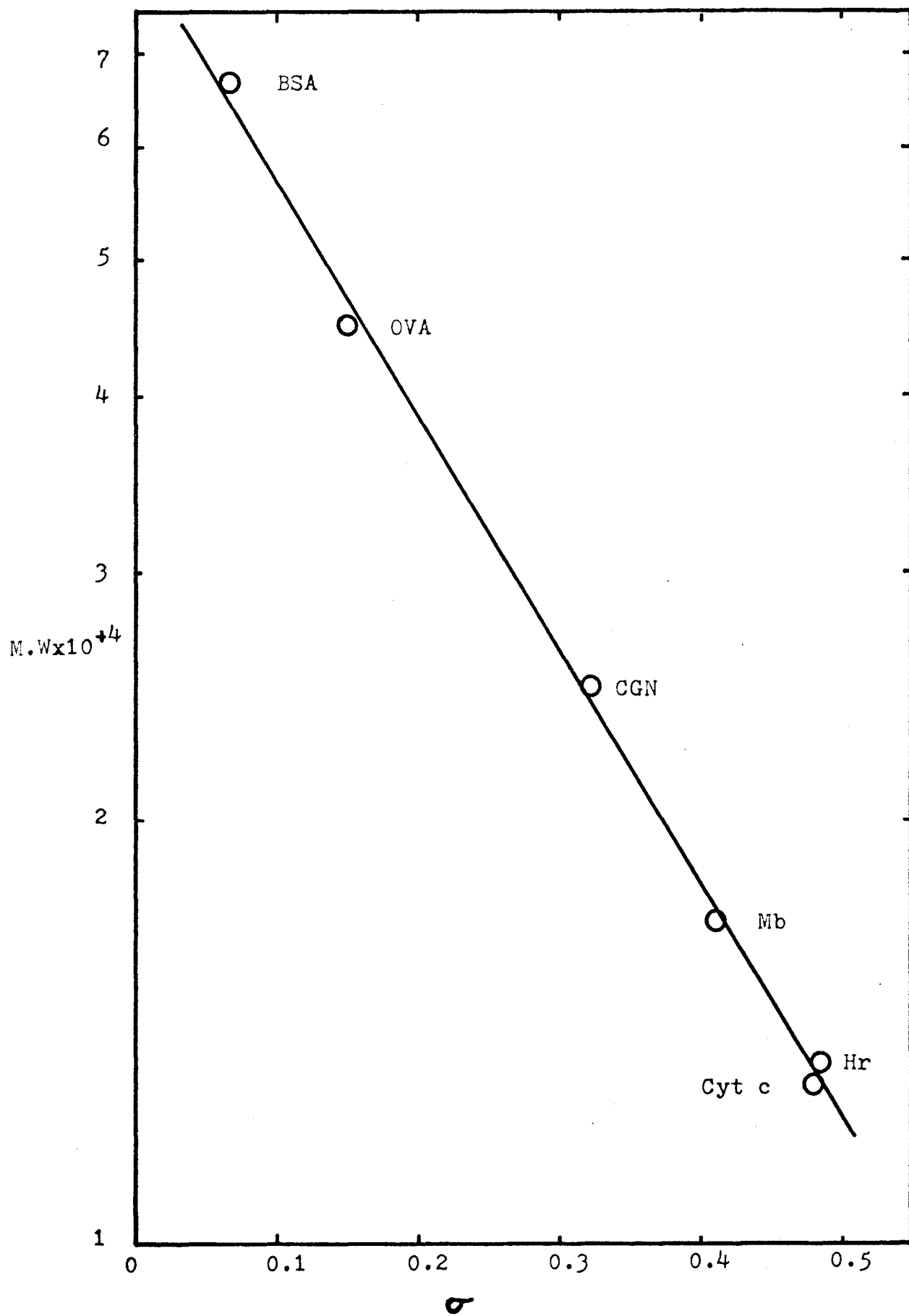
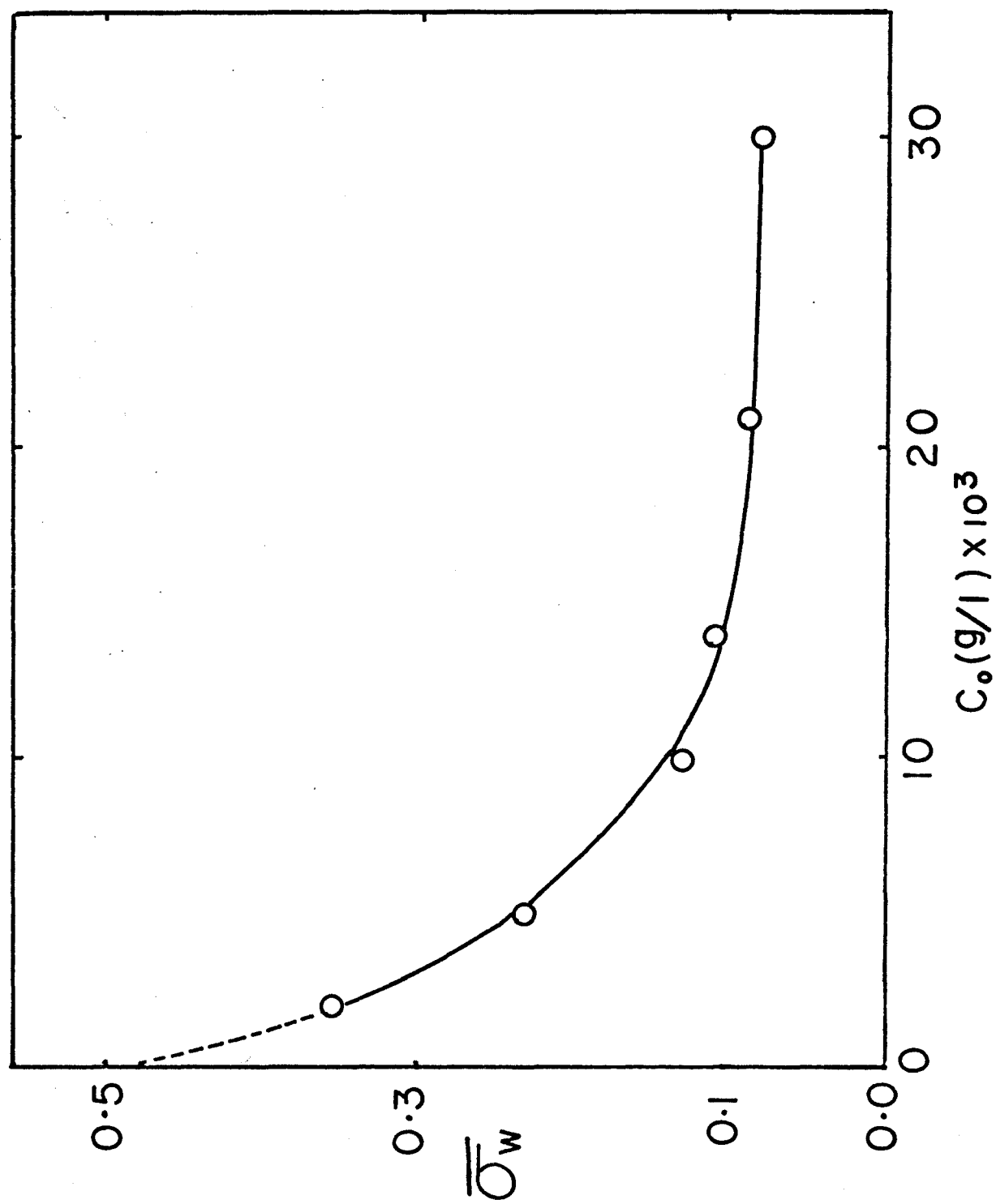


Figure 8. Plot of the weight average sieve coefficient against the oxyhemerythrin concentration (C_o) experiments on Sephadex G-75 column.



The second method involved direct measurement of the sieve coefficient for hemerythrin under conditions where the protein was fully dissociated. These conditions were at pH below 5.2 and a protein concentration below 20 $\mu\text{g}/\text{ml}$. The σ_1 obtained in this manner was 0.485 in excellent agreement with the value of 0.489 obtained by extrapolation as described previously.

The sieve coefficient for octameric hemerythrin was obtained directly, since at protein concentration above 150 $\mu\text{g}/\text{ml}$ oxyhemerythrin is fully associated (Keresztes-Nagy; Langerman et al, 1969). A 0.3 ml sample of oxyhemerythrin stock solution (about 6 mg/ml) was applied to the column such that the concentration of the protein eluted at the peak was about 200 $\mu\text{g}/\text{ml}$. The position of the peak was taken as the elution volume of octamer. Table III shows the sieve coefficients of octamer along with their standard error.

The sieve coefficients of dimer, trimer and tetramer of hemerythrin may be calculated from a knowledge of the calibration constant A and the sieve coefficient of monomer(σ_1). Ackers (1967) has developed a relationship,

$$\sigma_i = \sigma_1 - A \log i \quad (11)$$

where σ_i = sieve coefficient of species containing i monomeric subunits and i = number of monomeric subunits, i.e., 1, 2, 3 ... etc. The sieve coefficients for the i-mers calculated by Eq. (11) will have the same precision as that of the experimentally determined calibration constant and σ_1 . The sieve coefficients

TABLE III

Determination of the molecular sieve coefficient of octameric hemerythrin(σ_8) from zonal chromatography. A 0.3 ml oxyhemerythrin stock solution(about 6 mg/ml) was applied to a 1.5x60 cm column so that the concentrations of protein eluted at the peaks were always greater than 180 $\mu\text{g/ml}$.

Experiment	T $^{\circ}\text{C}$	pH	μ	σ_8	$(\sigma_8)^2$
1	25	7.00	0.01	0.0560	0.003136
2	25	7.00	0.01	0.0529	0.002798
3	25	5.70	0.01	0.0322	0.001037
4	25	7.00	0.01	0.0245	0.000600
5	25	5.80	0.01	0.0349	0.001218
6	25	5.80	0.01	0.0366	0.001340
7	5	5.30	0.01	0.0500	0.002500
8	15	5.80	0.01	0.0279	0.000778
9	5	5.46	0.01	0.0312	0.000973

$$\Sigma \sigma_8 = 0.3462$$

$$\Sigma (\sigma_8)^2 = 0.014380$$

$$\bar{\sigma}_8 = 0.0385$$

$$S^2 = \text{standard deviation} = \Sigma (\sigma_8)^2 - \bar{\sigma}_8 \cdot \Sigma \sigma_8$$

$$= 0.001063$$

$$\sigma_m^2 = S^2 / (n - 1) = 0.001063 / (9 - 1) = 0.000132$$

$$= \text{standard error}$$

$$\sigma_m = 0.01149$$

$$\bar{\sigma}_8 = 0.0385 \pm 0.0115$$

for monomer and several hypothetical intermediate species are given in Table IV.

TABLE IV
Sieve Coefficients of Intermediate
Species Calculated by Eq.(11)

$[\text{Hr}]_i$	Molecular Weight	σ_i
1	13,500	0.4835*
2	27,000	0.3012
3	40,500	0.2068
4	53,500	0.1361

* This is the mean value of σ_1
as given in Table VI.

Analysis of Elution Profiles Obtained by Frontal Chromatography

1. Determination of the centroid elution volume

An elution profile obtained by frontal chromatography is depicted by the solid line in Figure 9. The centroid volume(\bar{V}) of the ascending boundary is defined by Eq.(12):

$$\bar{V} = \frac{\int_0^{C_0} V dc}{C_0} \quad (12)$$

C_0 represents the protein concentration in the plateau region of the elution profile and it is identical to the protein concentration of the solution applied to the column. \bar{V} values can be determined by planimetry across the leading boundary. In ideal systems \bar{V} corresponds to the half height of the plateau, $C_0/2$. The dashed line in Figure 9 represents the first derivative of the elution profile as determined by the differentiator,

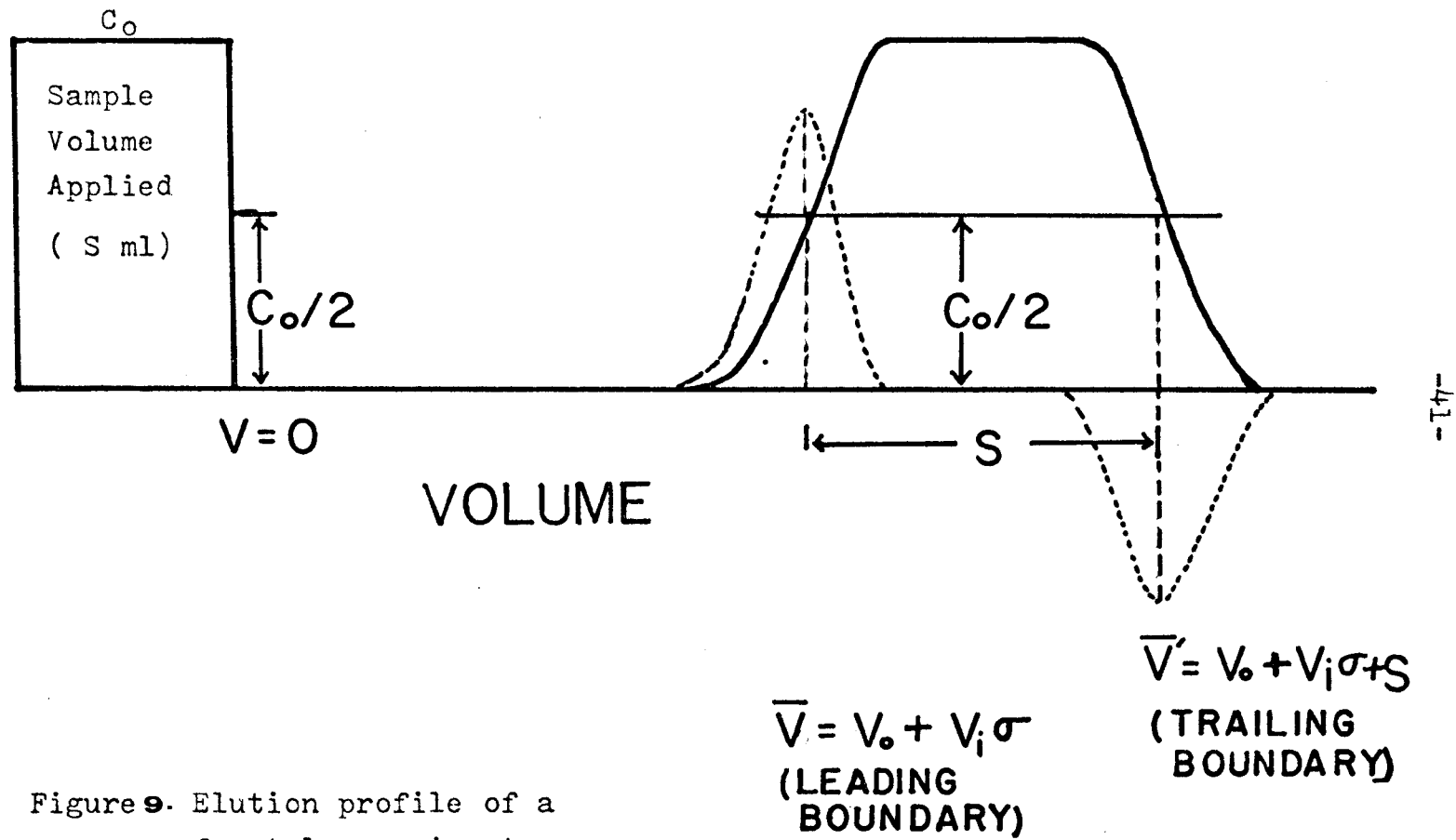


Figure 9. Elution profile of a frontal experiment

it can be seen that the position of the peak on the differential elution profile corresponds to the centroid volume(\bar{V}).

In a similar fashion the trailing boundary of the elution profile yields an elution volume, \bar{V}' , which is equal to the sum of the centroid volume and the sample volume(S) according to Eq.(13):

$$\bar{V}' = \bar{V} + S \quad (13)$$

From a knowledge of the sample volume and \bar{V}' from the descending boundary of the elution profile, one can calculate the centroid volume(\bar{V}).

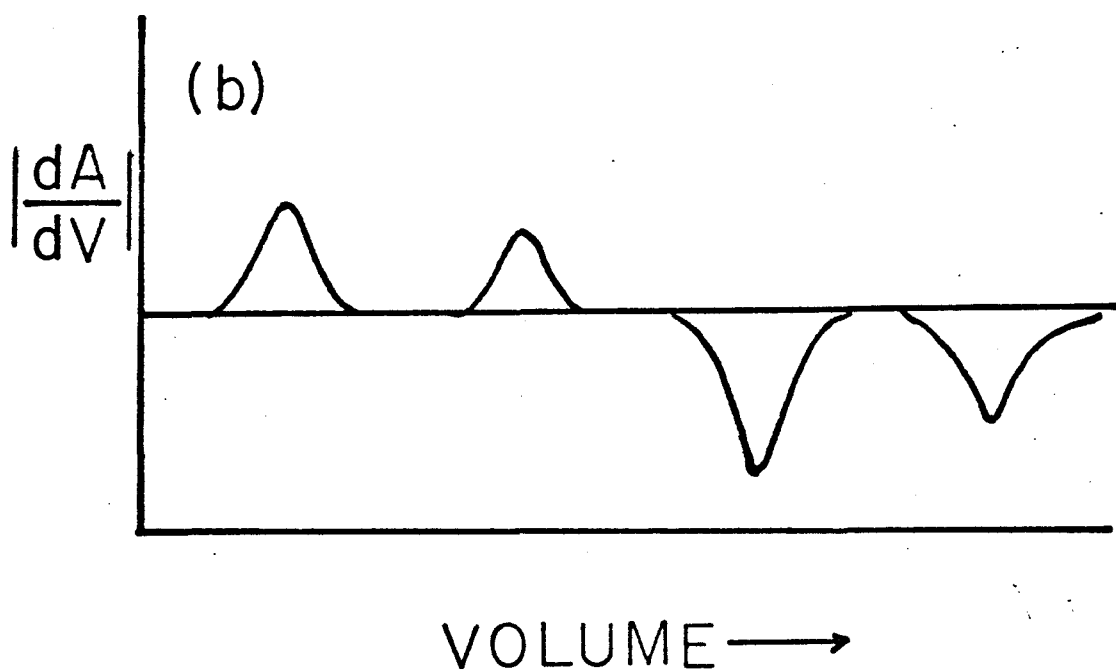
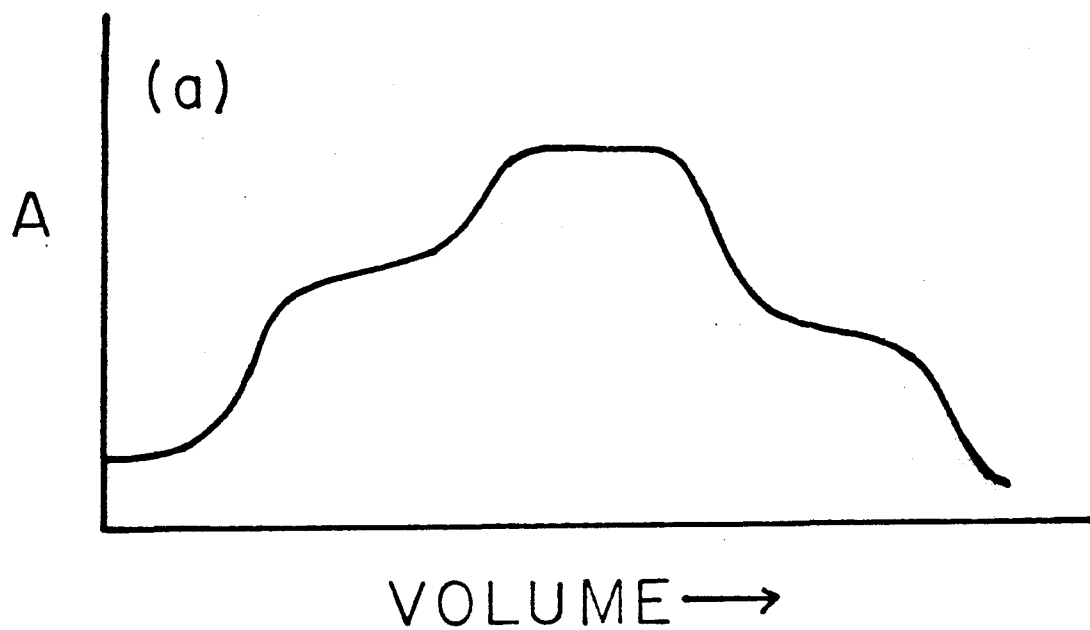
2. Calculation of sieve coefficients

In association-dissociation reactions which were slow compared with the time of the experiment the interacting species were completely separated from one another. The centroid volumes determined from the integral and differential elution profiles thus corresponded to the individual species originally present in the equilibrium mixture(Figure 10).

The sieve coefficients of these species were then obtained with the aid of Eq.(10). For rapid association-dissociation equilibration the observed elution profile showed only a single leading boundary. The sieve coefficient determined from the centroid volume of the boundary was a weight average sieve coefficient($\bar{\sigma}_w$) as defined by Eq.(14):

$$\bar{\sigma}_w = \frac{\sum_{i=1} \sigma_i C_i}{\sum_{i=1} C_i} = \frac{\sum_{i=1} \sigma_i K_i C_1^i}{C_o} \quad (14)$$

Figure 10. Integral and differential elution patterns of a slow association-dissociation reaction.



The individual sieve coefficients(σ_i) pertain to various aggregates (i-mer) and the C_i term represents their respective concentrations (expressed as weight per unit volume) in the plateau region. The concentration of i-mer is related to the concentration of the monomer (C_1) by the relationship,

$$C_i = K_i C_1^i \quad (15)$$

The weight average sieve coefficient is calculated for each run from the measured centroid volume, the void volume and the internal volume according to Eq.(10). As the total concentration of protein (C_o) increases, the various equilibria are shifted in favor of higher aggregates and this change is reflected in $\bar{\sigma}_w$ through Eq.(14).

3. Calculation of weight fraction of monomer(α_1)

The weight fraction of monomer(α_1) has been defined as the ratio of the concentration of monomer(C_1) to total protein concentration(C_o) as expressed in weight per unit volume. Determination of α_1 depends on the nature of the association-dissociation reaction. When the rate of the reaction was slow relative to the rate of separation on the column, the elution profile showed separate plateau regions corresponding to the various species present. The ratio of the plateau height of monomer to the total plateau height of the elution profile yielded α_1 .

For rapid association-dissociation reactions in which

species between monomer and n-mer were not present, α_1 could be calculated in several ways. First, from experimentally determined values of $\bar{\sigma}_w$, σ_1 and σ_n according to Eq.(16) (Ackers and Thompson, 1965) :

$$\alpha_1 = \frac{\bar{\sigma}_w - \sigma_n}{\sigma_1 - \sigma_n} \quad (16)$$

In this equation σ_n is the sieve coefficient of n-mer, σ_1 the sieve coefficient of monomer, and $\bar{\sigma}_w$ the weight average sieve coefficient. The value of α_1 could also be calculated according to Eq.(17):

$$\alpha_1 = \frac{\bar{M}_w - M_1}{M_n - M_1} \quad (17)$$

where M_n , M_1 and \bar{M}_w are the molecular weight of polymer, molecular weight of monomer and weight average molecular weight of the interacting macromolecular mixture. In the present investigation Eq.(17) was used to calculate α_1 when appropriate because the values of M_n and M_1 had been determined with great precision by ultracentrifugation(Keresztes-Nagy,1962) as well as from amino composition and sequence studies (Groskopf et al, 1966).

The calculation of α_1 for a rapid reaction is made more complex when significant amounts of species intermediate between monomer and n-mer are present. In that case, α_1 could be obtained by graphical integration according to the Steiner

Eq.(18) for an association reaction (1952):

$$\ln \alpha_1 = \int_0^c \left[\left(\frac{M_1}{\bar{M}_w} - 1 \right) / c \right] dc \quad (18)$$

I shall describe this procedure in detail in Chapter III.

4. Calculation of weight average molecular weight(\bar{M}_w)

For rapid association-dissociation reaction, \bar{M}_w is obtained from the experimentally determined values of $\bar{\sigma}_w$ with the aid of the calibration curve of σ versus $\log M$ (Figure 7).

For a slow reaction complete resolution of individual species is obtained by chromatography. The amount of each species present can be determined from the height of the various plateau regions in the frontal elution profile. \bar{M}_w may thus be evaluated with the aid of Eq.(19):

$$\bar{M}_w = \frac{\sum_{i=1}^n i M_i C_i}{C_o} \quad (19)$$

When only two species predominate, monomer and n-mer, Eq.(20) can be used to evaluate \bar{M}_w :

$$\bar{M}_w = \alpha_1 M_1 + (1 - \alpha_1) M_n \quad (20)$$

5. Determination of the Stoichiometry of the association-dissociation reactions

The trailing boundary of an elution profile can provide

information about the stoichiometry of a rapid association-dissociation reaction (Ackers and Thompson, 1965). As mentioned previously, for a non-ligand mediated reaction the bimodal behavior of the trailing boundary indicates a polymerization reaction. The volume corresponding to the minimum in the trailing boundary is related to the stoichiometry of the reaction (n) through Eqs. (21) and (22):

$$n = \frac{3v'_m - \sigma_1 - 2\sigma_n}{3v'_m - 2\sigma_1 - \sigma_n} \quad (21)$$

$$v'_m = \frac{V'_{\min} - S - V_o}{V_i} \quad (22)$$

V'_{\min} represents the volume which corresponds to the position of the minimum in the descending boundary of the elution profile. V_o , S and V_i have their usual meanings. The v'_m then represents the position of the minimum corrected for column volume and sample volume. The most reliable values of V'_{\min} were determined from the differential elution profiles which were obtained with the aid of the curve differentiator. It should be emphasized that Eq. (21) is only valid for non-ligand-mediated reactions.

6. Calculation of equilibrium constants

For simple association-dissociation equilibria in which only monomer and n-mer are present one can easily calculate equilibrium constants with the help of either equation (23a)

or equation (23b) (Ackers and Thompson, 1965) :

$$K = \frac{\alpha_1^n C_o^{n-1}}{1 - \alpha_1} \quad (23a)$$

$$K = \frac{n (C'_{\min})^{n-1}}{\lambda + (1 + \lambda/n)^{n-1}} \quad (23b)$$

In these equations α_1 and C_o have their usual meanings. C'_{\min} corresponds to the protein concentration at the minimum in the trailing boundary, and λ is given by Eq.(24) :

$$\lambda = \frac{\sigma_1 - V'_{\min}}{V'_{\min} - \sigma_n} \quad (24)$$

Of the two methods of determining K, Eq.(23a) is the more useful. It yields the most reliable values of K and is used exclusively where applicable.

For more complex association-dissociation reactions in which significant amounts of species intermediate between monomer and n-mer were present, Eq.(23a) and (23b) could not be used to calculate meaningful equilibrium constants. For such a system , the overall equilibrium constant for the reaction is given by the product of the individual formation constants for the various intermediate species. I shall elaborate on this further in Chapter III.

CHAPTER III

EXPERIMENTAL RESULTS

Calibration of Sephadex Columns

The sieve coefficients (σ) of the marker proteins, derived from the elution volume (according to Eq.(10)), and the logarithm of the corresponding molecular weights are listed in Table I. These data were found to obey the linear relationship given by Eq. (9) within the exclusion limit (71,000) of Sephadex G-75 (Andrews, 1965). The best straight line together with calibration constants A and B were obtained by the method of least squares with the aid of an Olivette Underwood Programma 101 computer. The values of A and B were found to be 0.5800 and 2.8613, respectively. They agreed closely with those reported by Henn and Ackers (1968). The effect of temperature on the calibration curve was also studied. Whitaker (1963) had observed that the elution volume and V_e/V_0 were slightly affected by temperature. The present work confirmed that elution volumes did vary with temperature. However, as shown in Table V, the parameter, V_e/V_0 and the sieve coefficient (σ) for the marker substances were much less sensitive to temperature variation. Anyway, calibration curves were obtained at several temperatures so that they could be used to measure the weight average molecular weight of interacting hemerythrin

TABLE V

Effect of temperature on the elution volume of marker substances on Sephadex G-75 column

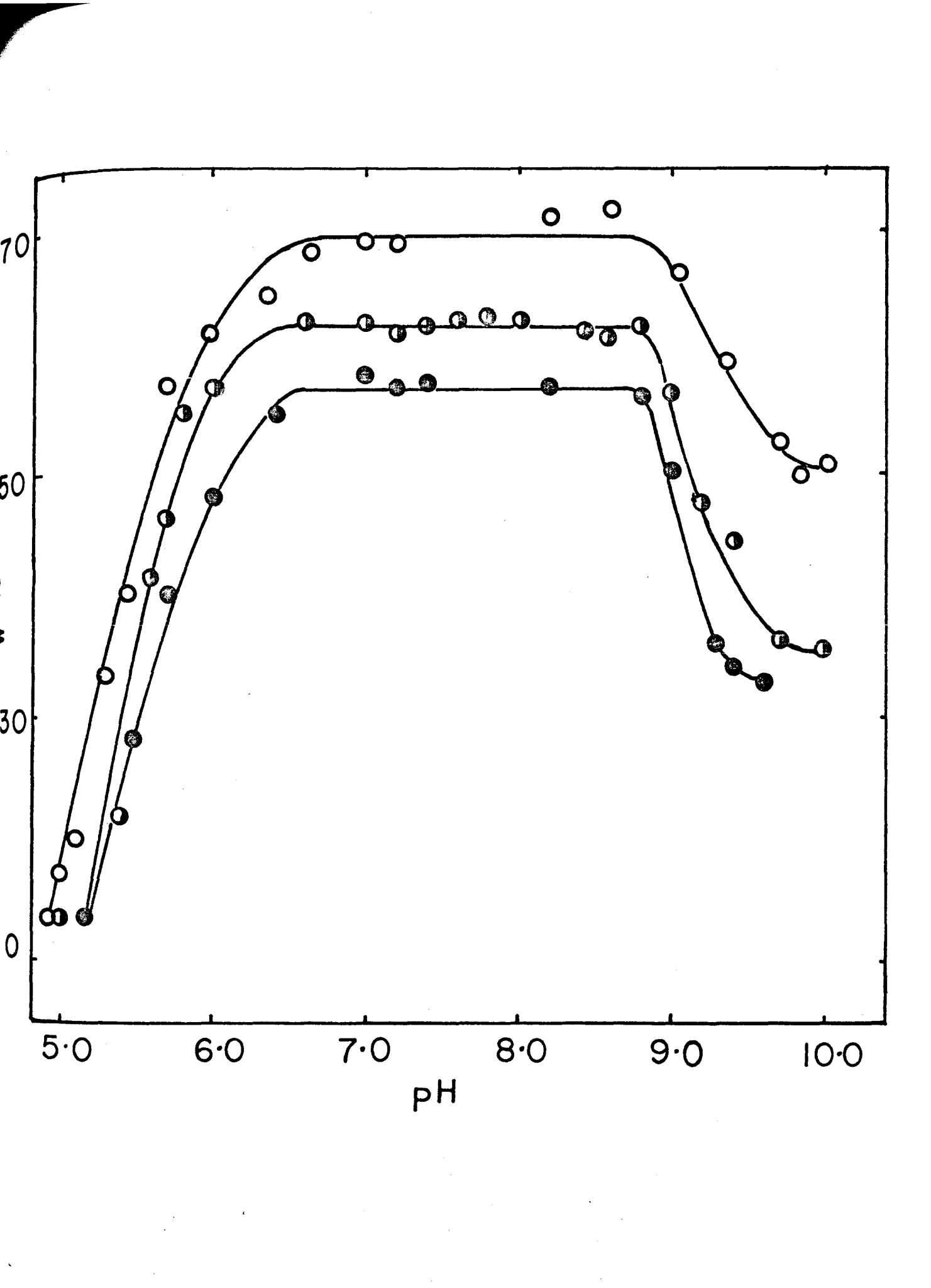
Marker	T °C	V _e (ml)	V _e / V _o	σ
B.D.	10	22.48	1.00	0.000
Hr ₈	10	25.02	1.11	0.058
BSA	10	25.61	1.14	0.072
CGN	10	37.10	1.65	0.339
K ₂ CrO ₄	10	66.00	2.94	1.000
B.D.	20	22.40	1.00	0.000
Hr ₈	20	24.89	1.11	0.057
BSA	20	25.37	1.13	0.068
CGN	20	36.80	1.64	0.330
K ₂ CrO ₄	20	66.06	2.95	1.000
B.D.	25	23.52	1.00	0.000
Hr ₈	25	26.08	1.10	0.056
BSA	25	26.53	1.13	0.066
CGN	25	38.23	1.63	0.322
K ₂ CrO ₄	25	69.20	2.94	1.000

system at the appropriate temperature. A representative calibration curve at 25° C. is shown in Figure 7. Finally, as an added precaution, the values of V_0 , V_i , A and B were periodically redetermined for the Sephadex columns in order to ascertain that there were no changes in the properties of these columns with use.

pH Dependence of Dissociation of Oxyhemerythrin at Three Temperatures

All the experiments, with the exception of those at low pH, were carried out at a constant protein concentration of 30 $\mu\text{g/ml}$ in 0.01 μ Tris-cac buffer. At low pH higher protein concentrations were also used in order to determine the degree of dissociation more accurately. The integral and differential elution profiles were simultaneously recorded. Figure 11 shows the weight average molecular weight of a hemerythrin solution (30 $\mu\text{g/ml}$) as a function of pH at three different temperatures. These were typically flat-top bell shape profiles indicating that the polymer was dissociated in acidic and basic solutions while the native protein was most stable in the region of neutral pH. Although not apparent from Figure 11, there were qualitative as well as quantitative differences in the dissociation behavior of hemerythrin in different pH regions. Below pH 6.4, both the leading and the trailing boundaries of the elution profiles showed two well defined plateau regions as

Figure 11. Dependence of the weight average molecular weight, \bar{M}_w of oxyhemerythrin upon pH at constant protein concentration of 30 $\mu\text{g/ml}$. The buffer was 0.01 μ Tris-cacodylate . $T = 5^\circ$ (open circles); $T = 25^\circ$ (half-filled circles); $T = 30^\circ$ (filled circles).



shown in Figures 12a and b. The centroid elution volumes of the fast and the slow moving components were measured and were used to calculate the sieve coefficients of the two species. From the knowledge of the sieve coefficients the two species were identified as monomer and octamer. The appearance of the two plateau regions could have either resulted from the presence of a denatured or "crippled" species which could not participate in the reassociation reaction or from a slow equilibration between the interacting species. The degree of dissociation was found to increase with decreasing pH as shown in Figures 12a and b. At a protein concentration of 30 $\mu\text{g/ml}$ and pH values less than 5.2, the polymeric hemerythrin was completely dissociated into monomeric subunits as indicated by curve cc of Figure 12a and as shown in Tables VII and VIII.

The elution profiles for the reaction between pH 6.6 and 7.4 are shown in Figure 13. In this pH region, the elution diagram consisted of a single sharp leading boundary, a plateau region and an unresolved bimodal trailing boundary. If one assumes that only two species were in equilibrium, then according to the Gilbert theory such an elution pattern could be characteristic of a rapidly polymerizing system, in which the stoichiometry (n) of the reaction is greater than 2. In addition, the dissociation reaction appeared independent of

Figure 12a. Elution profiles obtained in the acidic pH regions. The buffer was Tris-cacodylate (0.01 μ), $T = 25^{\circ}$. Curve aa: pH 6.00; Curve bb : pH 5.60; Curve cc : pH 5.20 .

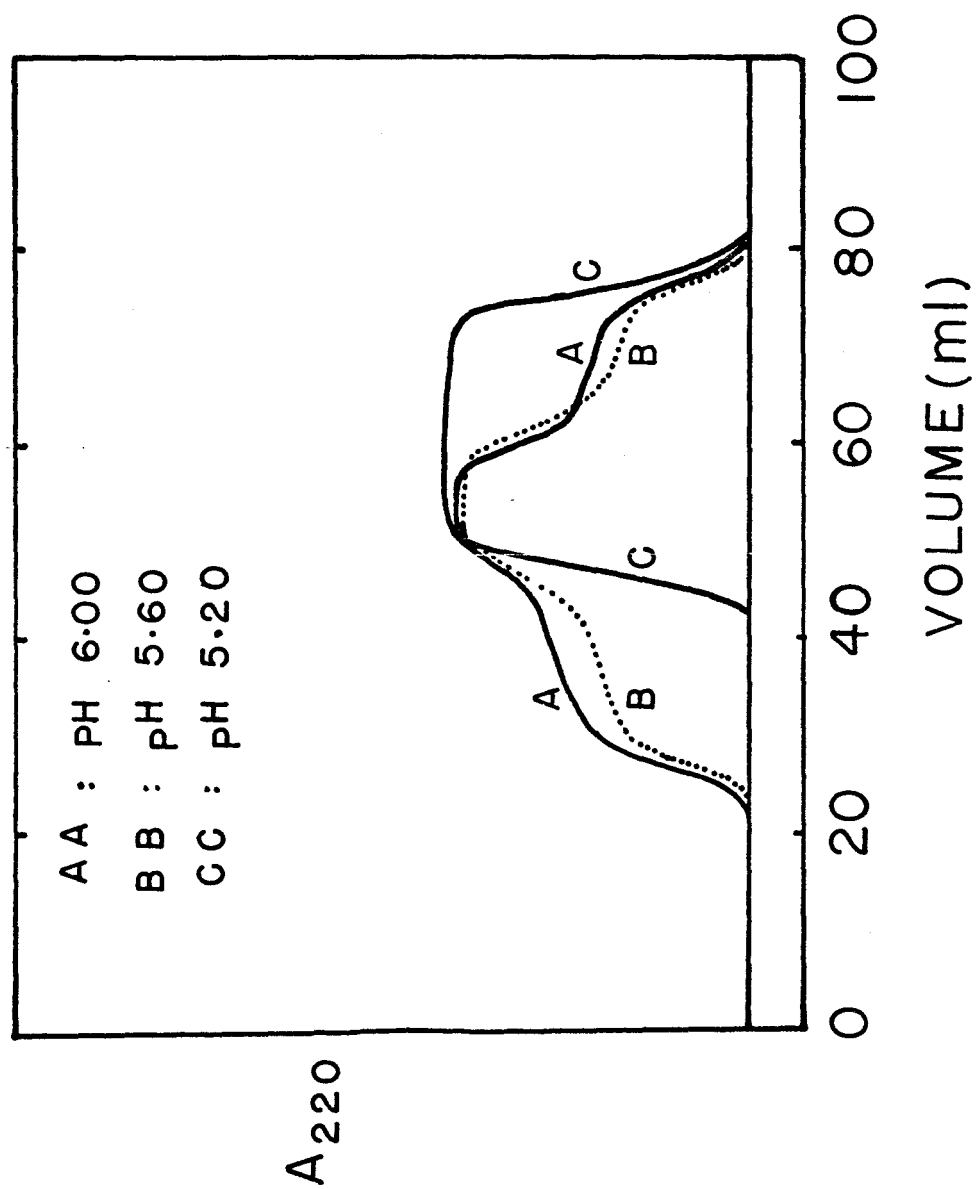


Figure 12b. Integral and differential elution diagrams depicting the trailing boundary as obtained at pH 5.8 and 30 μ g/ ml protein concentration. Other conditions were the same as those in Figure 12a.

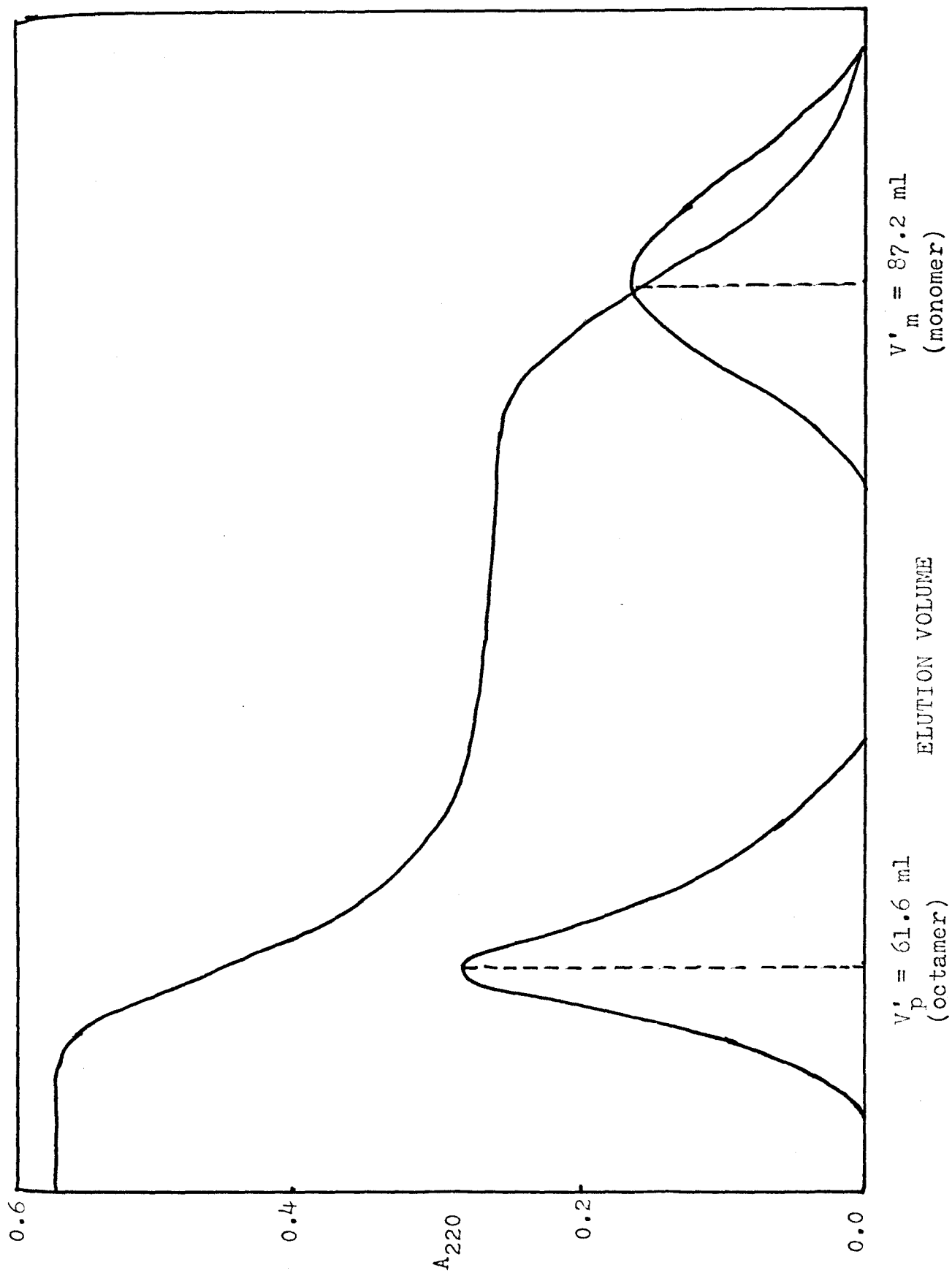


Figure 13. Typical elution diagram of oxyhemerythrin at neutral pH, 0.01 μ Tris-cacodylate buffer at 25°.

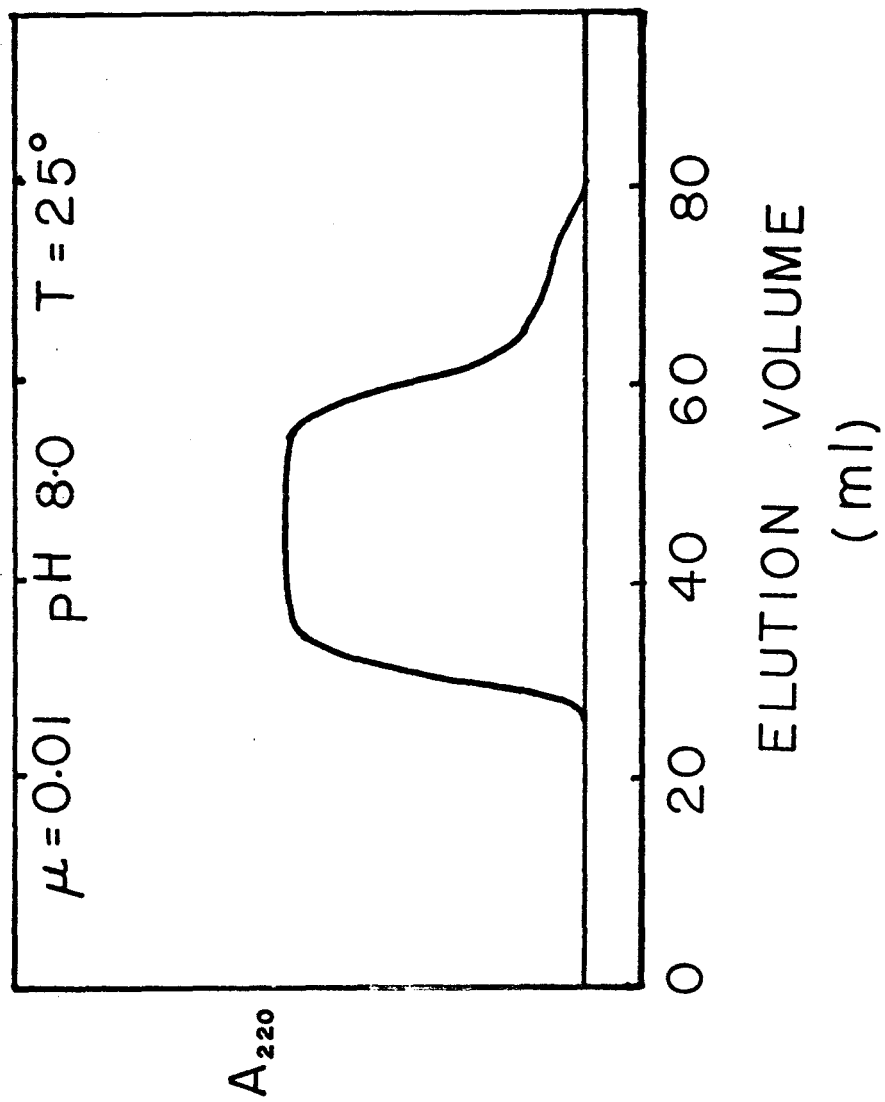


Figure 14. Typical elution patterns obtained between pH 8.0 and 8.8 . The protein concentration was 30 $\mu\text{g/ml}$ in 0.01 μ Tris-cacodylate buffer and at 25 $^{\circ}$.

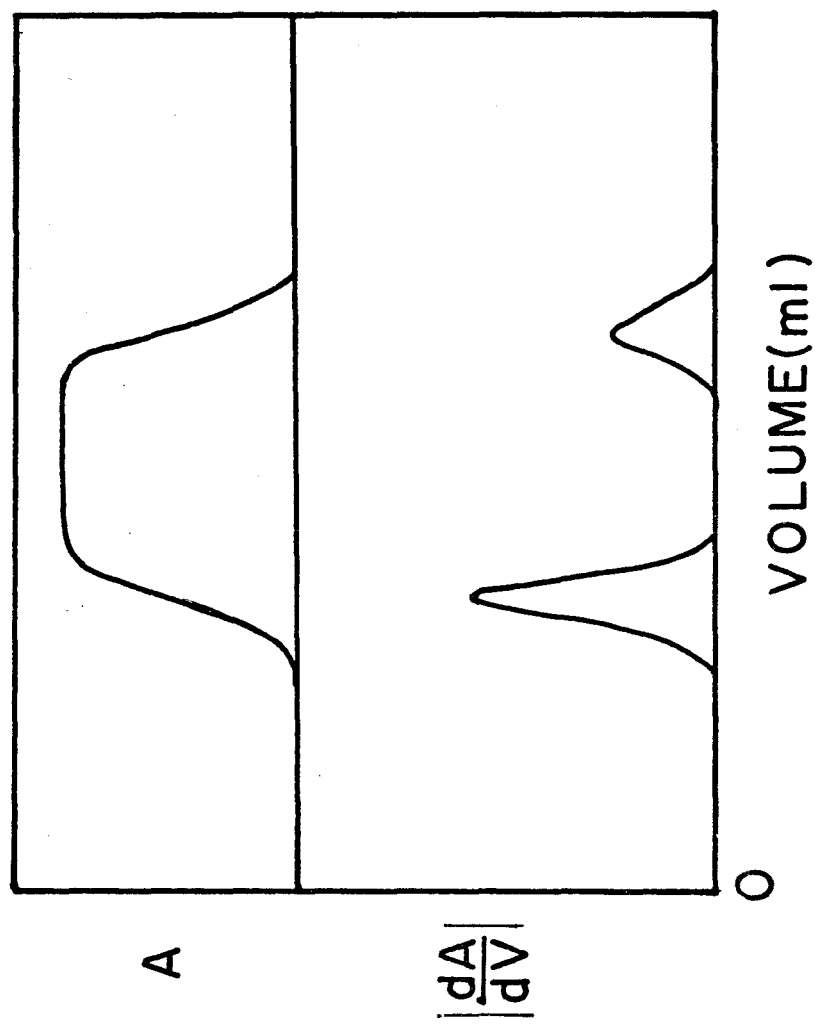


Figure 15a. Elution diagrams obtained between pH 9.0 and 9.7. The oxyhemerythrin concentration was 30 $\mu\text{g/ml}$ in 0.01 μ Tris-cacodylate buffer and at 25 $^{\circ}$.

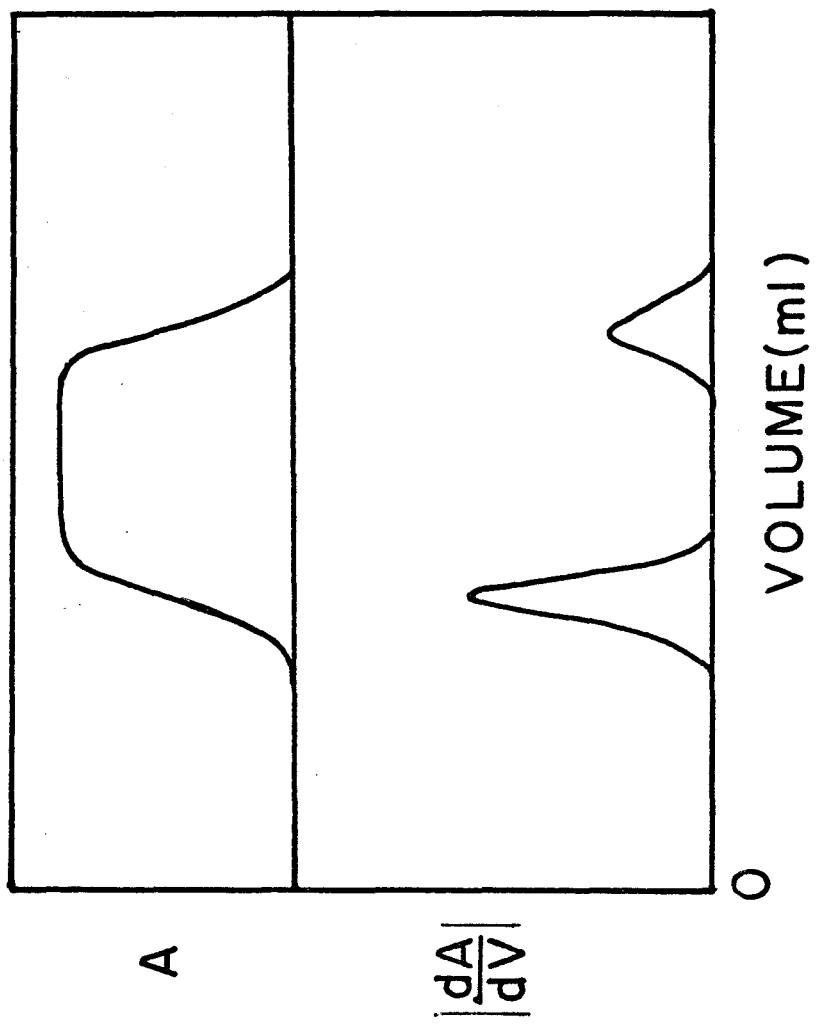


Figure 15a. Elution diagrams obtained between pH 9.0 and 9.7. The oxyhemerythrin concentration was 30 $\mu\text{g/ml}$ in 0.01 μ Tris-cacodylate buffer and at 25 $^{\circ}$.

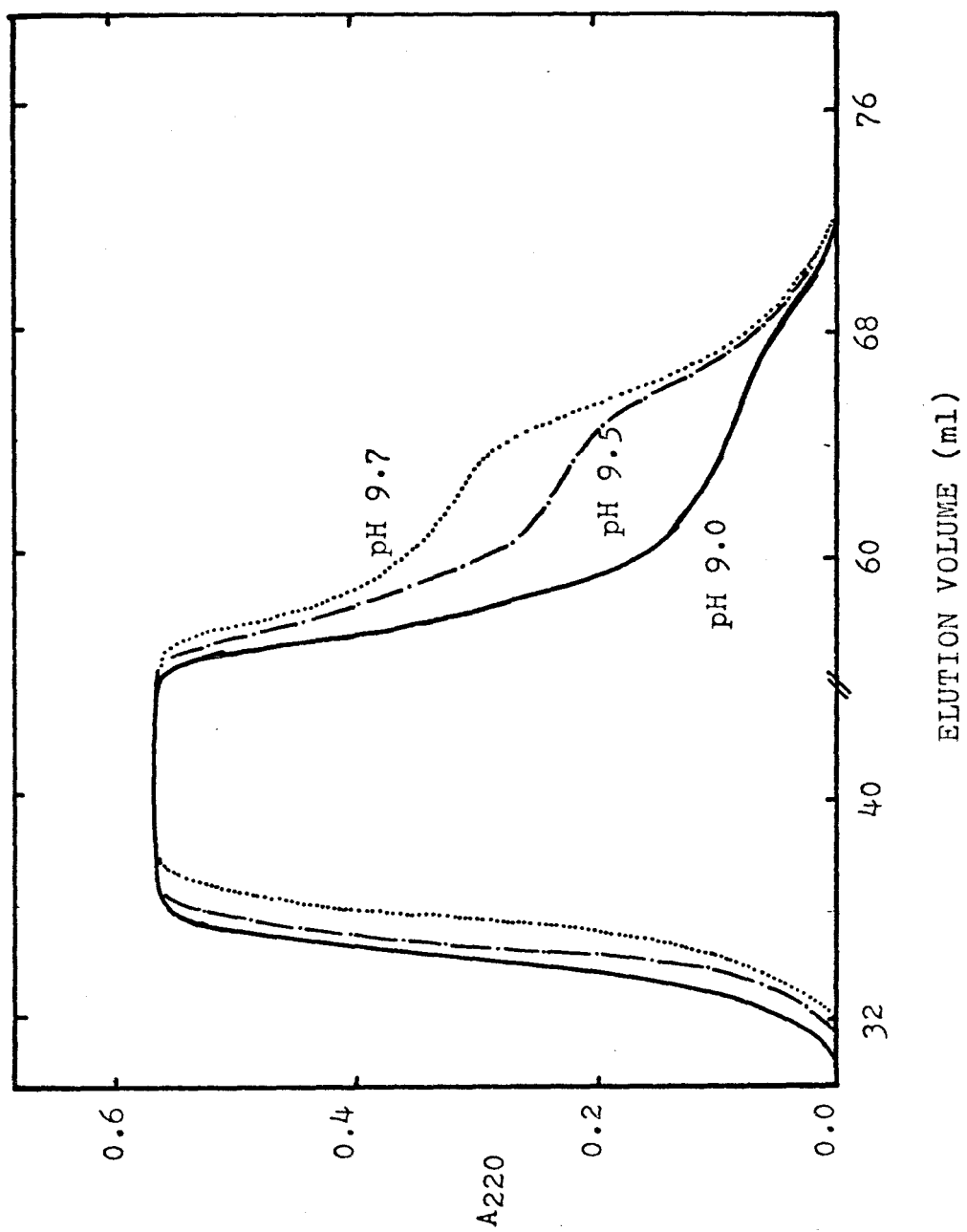
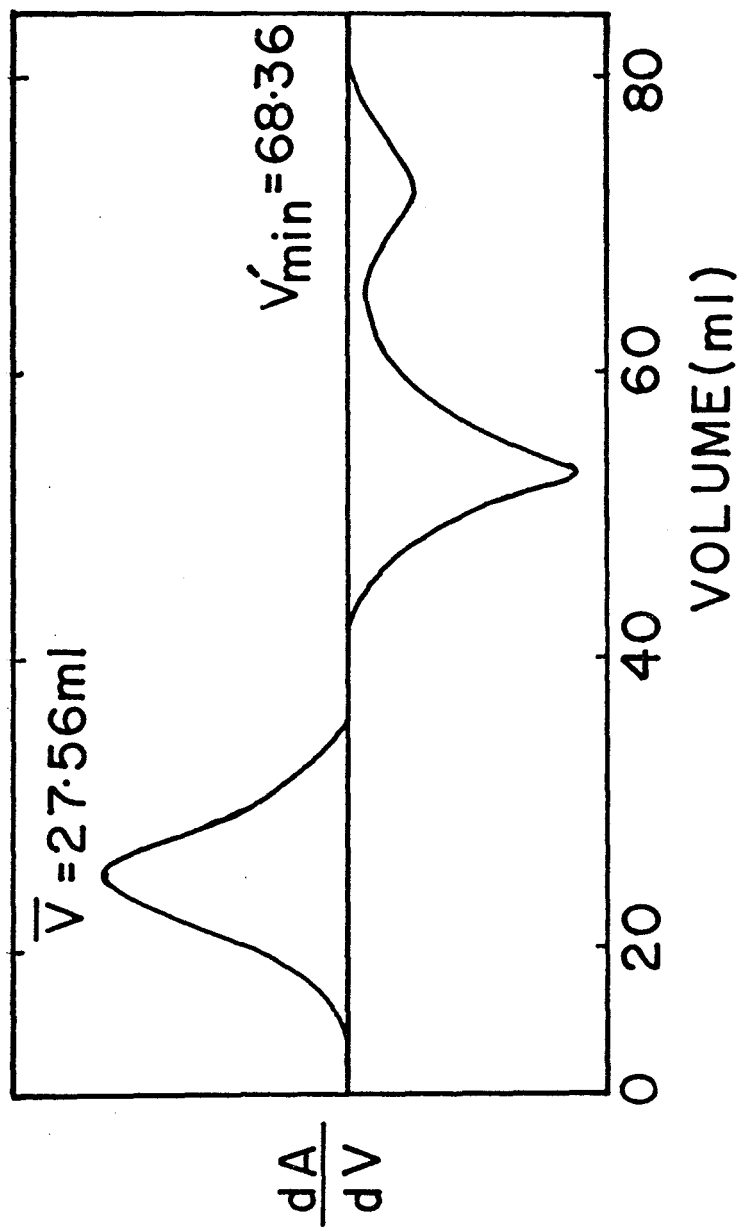
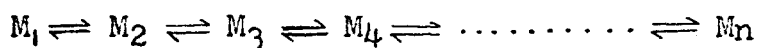


Figure 15b. Differential elution diagrams obtained
at pH above 9.0.



the pH between 6.6 and 7.4.

In contrast to the patterns observed in the neutral pH, the elution profile obtained between pH 8.0 and 8.8 showed a single leading boundary, a plateau region and an unimodal trailing boundary (Figure 14). Such a pattern indicates a dimerization reaction. However, the actual mechanism of the dimerization needed to be further explored because several processes could give rise to an unimodal trailing boundary. These include simple dimerization, consecutive dimerization, and continual polymerization of the type



The elution profiles of the reaction occurring between pH 9.0 and 10.0 resembled those obtained in the neutral pH regions and they are shown in Figure 15a and b.

Inapplicability of Cann's Theory in the Four pH Regions

The changes in weight average molecular weight as a function of pH as shown in Figure 11 suggest that the degree of dissociation increase at both the acidic and alkaline pH regions, but is independent of pH in the neutral pH and between pH 8.0 and 8.8. Thus, it is only necessary to consider whether the association-dissociation reactions are proton-mediated in the acidic and alkaline pH regions. The reaction in the acidic pH range has been already shown to be slow. For such a slow

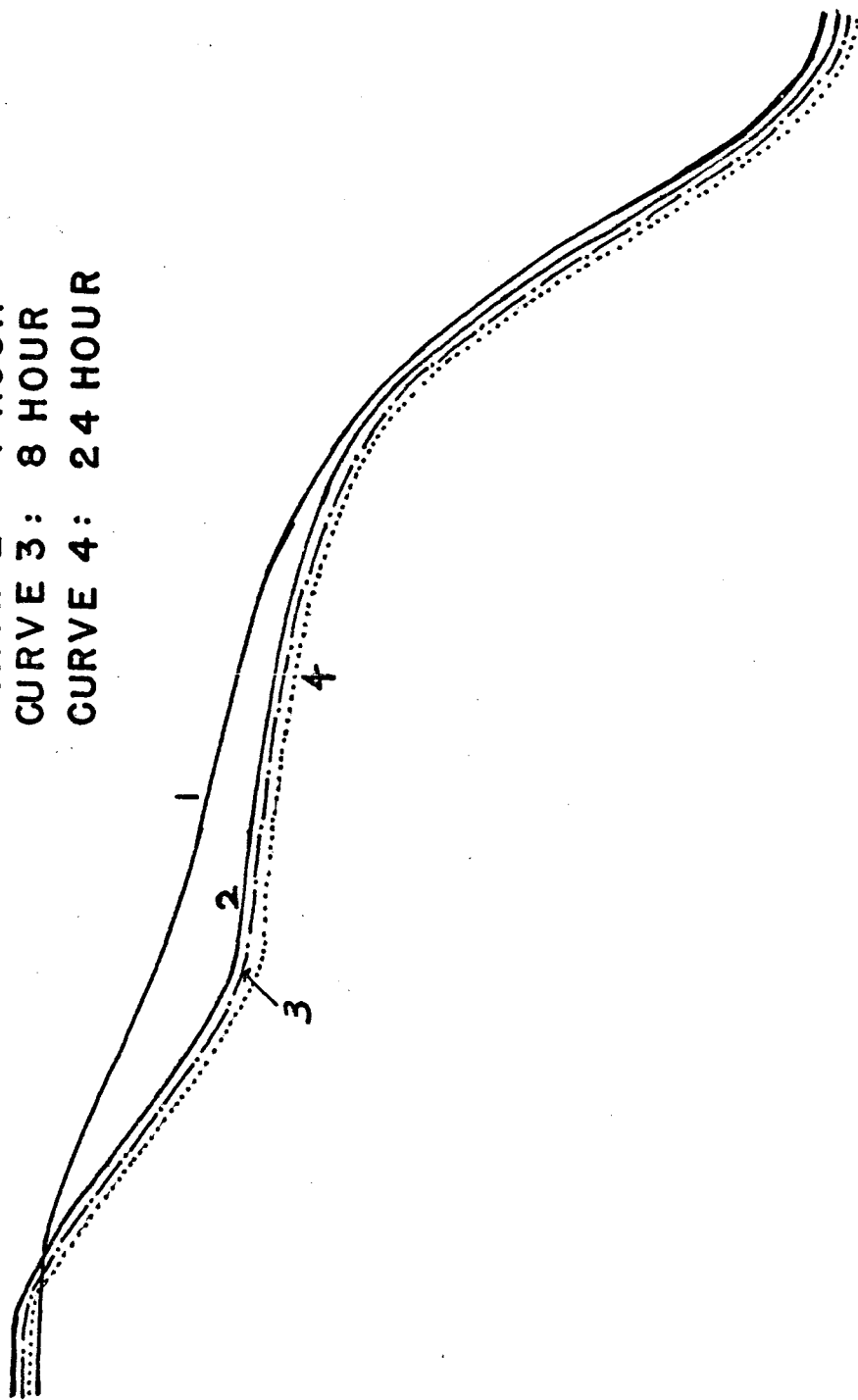
reaction, Cann's theory as explained in chapter 1 is not applicable. In the alkaline region, the buffering capacity of the buffer system was sufficiently large relative to the protein concentration to effectively prevent the formation of a pH gradient along the length of the column. One can thus safely apply Gilbert's theory to the interpretation of the elution patterns and to the calculation of equilibrium constants in the pH range from 4.8 to 9.8.

Association-Dissociation Reaction Between pH 4.8 and 6.4
at 5°, 25° and 30°.

As indicated previously, the rate of the association - dissociation reaction of hemerythrin was slow between pH 4.8 and 6.4. Because hemerythrin solutions in this pH range must be allowed to reach equilibrium before they are applied to Sephadex columns, it was necessary to obtain an estimate of the time required for equilibration. This was accomplished as follows: A 300 ml oxyhemerythrin (25 µg/ml) solution was prepared in 0.01 M Tris-cac at pH 6.0. Immediately, 50 ml of this solution was drawn into the sample loop and 32 ml applied to the column. The elution diagram was recorded. This was taken as the zero time elution pattern. The remaining solution was maintained in the refrigerator. At intervals of 4, 8 and 24 hours additional aliquots were removed, warmed to room temperature, and applied to the column. The elution profiles were again recorded.

Figure 16. Estimation of the rate of attainment of equilibration of oxyhemerythrin in the acidic pH region. The protein concentration was 25 $\mu\text{g/ml}$ in 0.01 μ Tris-cacodylate buffer. Curve 1 : Elution diagram of an oxyhemerythrin solution at pH 6.00 with zero hour of equilibration; Curve 2 : 4-hour equilibration ; Curve 3 : 8-hour equilibration; Curve 4 : 24-hour equilibration.

CURVE 1: 0 HOUR
CURVE 2: 4 HOUR
CURVE 3: 8 HOUR
CURVE 4: 24 HOUR



ABSORBANCE

VOLUME

Figure 16 shows the leading boundaries of the elution profiles observed at 0, 4, 8, and 24 hours. For the samples with less than four hour equilibration, the plateaus were diffuse and ill-defined (curve 1). However, the patterns obtained after 8 and 24 hour equilibration appear similar to that of the 24 hour sample (curves 2,3 and 4). The equilibrium between the subspecies appears to be established between 4 to 6 hours. Consequently, for all the experiments in the acidic pH range, the protein solutions were allowed to equilibrate for 5 hours in the refrigerator prior to application to the Sephadex columns.

Reversibility of the Association-Dissociation Reaction at Acidic pH

The elution profiles obtained for the reactions occurring at pH's below 6.4 showed two separate plateau regions both in the leading and the trailing boundaries (Figures 12a and b). The appearance of these separate plateaus may be due to either of two reasons. The first is the presence of a denatured or "cripple" species in the reaction mixture that could not participate in the association-dissociation reaction. This species would behave as a noninteracting and independent entity and be separate from the rest of the interacting molecules during differential transports on the column. The other possibility is a slow equilibration between the interacting species as

compared to the time for passage of these species through the column. To verify the latter possibility it was necessary to demonstrate the reversibility of the association-dissociation reaction. In one series of experiments the relative amounts of fast and slow moving component was studied as a function of protein concentration. Three sample solutions containing 30 $\mu\text{g/ml}$, 15 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ of oxyhemerythrin in 0.01 M Tris-cac buffer at pH 6.00, were equilibrated for six hours. Each solution was then applied to the same column and their elution profiles were recorded and compared. The centroid volumes of the fast and the slow moving components were essentially the same at these protein concentrations. Only the relative amounts of the two components varied with protein concentrations. This clearly indicates that the species are in dynamic equilibrium even though the rate of equilibration is slow. A second series of experiments were performed in order to demonstrate that the elution profiles obtained at low pH were readily reversed at higher pH. The first step was to obtain an elution profile for a hemerythrin solution at pH 7.00. This elution profile would then serve as a reference. Then, 200 ml of an identical hemerythrin solution was prepared at pH 6.00 and equilibrated in the refrigerator for six hours. An aliquot of this solution was applied to a second column previously equilibrated with pH 6.00 Tris-cac buffer and the elution profile recorded. The

remaining protein solution at pH 6.00 was adjusted to pH 7.00 by adding solid Tris. After an additional six hours in the refrigerator an aliquot of this solution was removed and applied to the Sephadex column at pH 7.00 and the elution profile recorded. The remaining portion of the protein solution was kept refrigerated over night and an additional aliquot applied to the column at pH 7.00. The elution profile was again recorded. All four elution profiles are presented in Figure 17. Only the oxyhemerythrin solution at pH 6.00 shows two separate plateaus (curve 2). The two profiles (curves 3 and 4) obtained for the protein solutions which had been adjusted from pH 6.00 to 7.00 appear identical with the elution pattern for the protein solution which had no prior exposure to pH 6.00 (curve 1).

The same type of reversibility experiments were performed for oxyhemerythrin solution at pH 5.00. At 30 $\mu\text{g/ml}$ protein concentration, oxyhemerythrin was completely dissociated into monomers and only single leading and trailing boundaries were observed. The centroid elution volumes of these boundaries corresponded to that of the pure monomer (curve bb of Figure 18). Curve aa is the elution profile for protein solution prepared at pH 7.00 while curve cc was that of the protein solution at pH 7.00 after prior exposure to pH 5.00. These experiments demonstrate that monomeric oxyhemerythrin generated at low pH reassociated and thereby participate in a rapid

Figure 17. Reversibility test for the association-dissociation reaction at pH 6.00. The protein concentration was 30 μ g/ml in 0.01 Tris-cacodylate buffer. Curve 1 : the leading boundary of an oxyhemerythrin solution in 0.01M pH 7.00 buffer; Curve 2 : Elution diagram of an oxyhemerythrin solution in pH 6.00 buffer after the sample having been kept equilibrated for six hours; Curve 3 : Elution diagram of the protein solution readjusted from pH 6.00 to 7.00, and equilibrated for six hours before applied to column. Curve 4 : the same solution as in case 3 except the sample was equilibrated over night before it was applied to column.

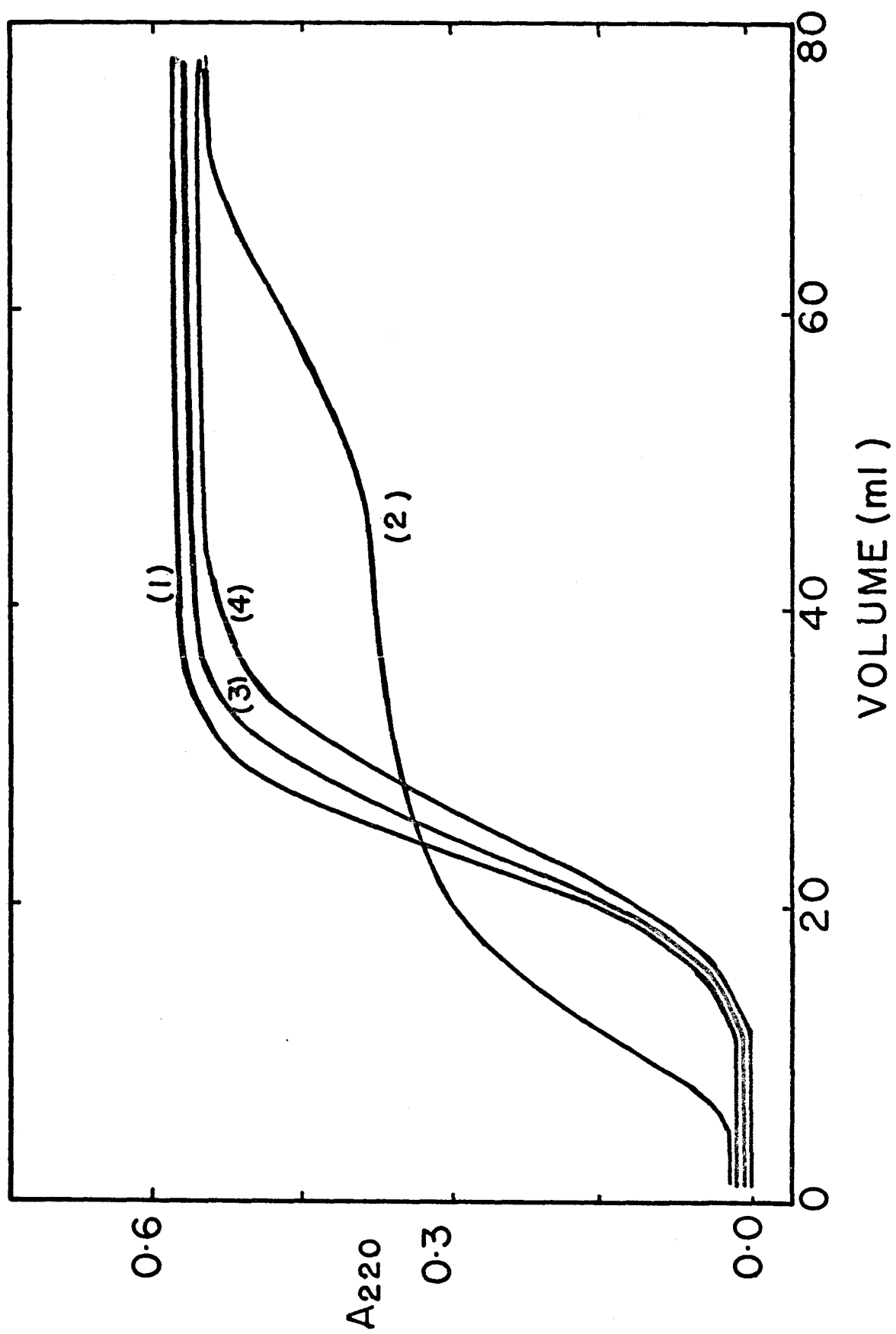
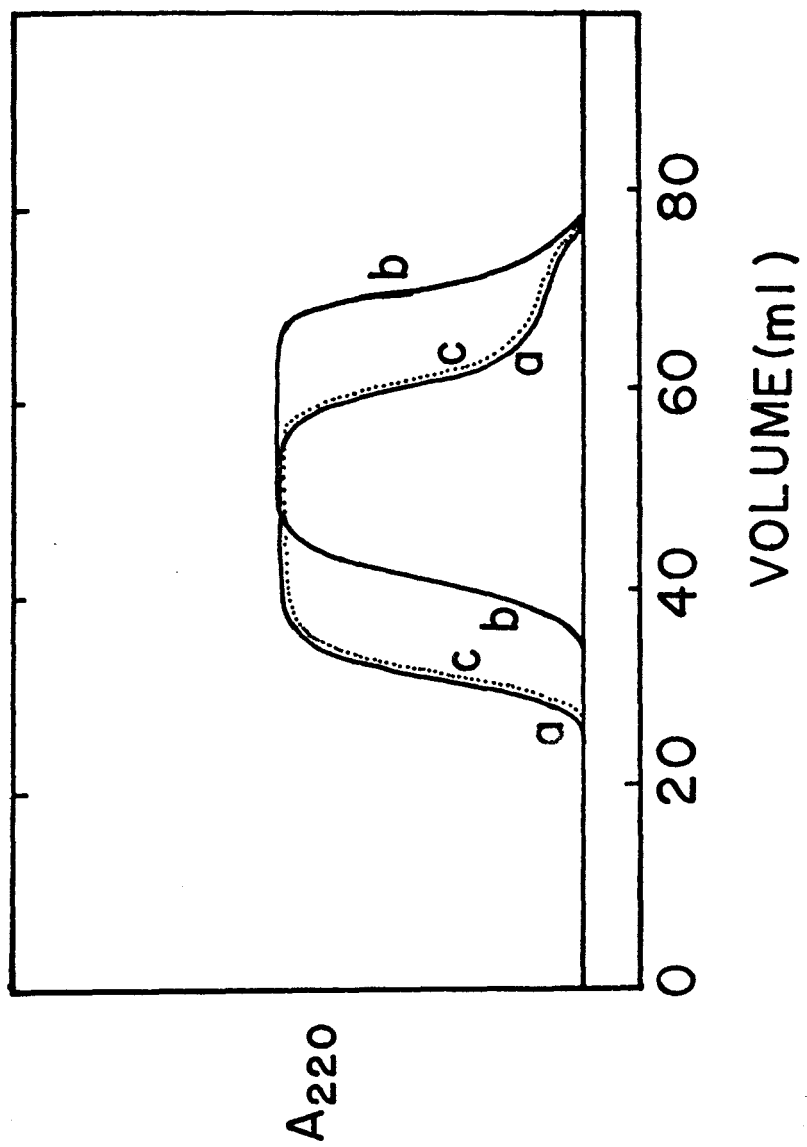


Figure 18. Reversibility test for the association-dissociation reaction at pH 5.00. Curve aa : Elution pattern at pH 7.00, 25°. Curve bb : protein solution in pH 5.00 buffer for six hours in refrigerator and then applied to column at 25° ; Curve cc : Elution profile of protein solution readjusted from pH 5.00 to 7.00 and kept in refrigerator for additional six hours before it was applied to column.



monomer \rightleftharpoons octamer equilibrium at neutral pH.

Determination of Equilibrium Constants at Acidic pH

We have just shown that the presence of two well separated boundaries in the elution profile at low pH was due to slow reequilibration between the interacting species, since the reaction has been proven to be reversible. The nature of the subspecies may be deduced from the knowledge of their sieve coefficients. Table VI summarizes the sieve coefficients obtained from the trailing boundaries under various conditions of pH and temperature. The mean sieve coefficient for the fast moving (σ_f) and that of the slow moving (σ_s) components were found to be 0.0404 ± 0.0067 and 0.4835 ± 0.0153 , respectively. When these values are compared to the sieve coefficients of the pure monomer and octamer (Tables III and IV) obtained from independent experiments, the fast and slow components are identified as the octameric and monomeric hemerythrin.

A knowledge of the nature of the species in equilibrium allows calculation of a dissociation constant for the reaction. Equilibrium constants are tabulated in Tables VII, VIII and IX. The pH dependence of the logarithm of the dissociation constants from 4.8 to 7.4 at 25° is shown in Figure 19a. Two kinds of information may be derived from the plot of $\log K_{eq}$ versus pH.

TABLE VI

Sieve Coefficients of Octameric and Monomeric Hemerythrin Obtained from the Trailing Boundaries of Frontal Elution Diagrams. $C_0 = 30 \mu\text{g/ml}$ in 0.01μ Tris-Cacodylate Buffer.

T °C	pH	σ_1	$(\sigma_1)^2$	σ_8	$(\sigma_8)^2$
30	6.41	0.4622	0.213629	0.0334	0.001116
5	6.36	0.4918	0.241867	0.0434	0.001884
10	6.00	0.4720	0.222784	0.0350	0.001225
10	6.00	0.4938	0.243838	0.0425	0.001806
25	5.80	0.4700	0.220900	0.0340	0.001156
15	5.80	0.4607	0.212244	0.0410	0.001681
25	5.70	0.4912	0.241277	0.0435	0.001892
20	5.70	0.4974	0.247407	0.0415	0.001722
5	5.70	0.5056	0.255631	0.0342	0.001170
25	5.30	0.4900	0.240100	0.0544	0.003069
25	5.00	0.4840	0.234256	-----	-----

$$\bar{\sigma}_1 = 0.4835 \pm 0.0153$$

$$\bar{\sigma}_8 = 0.0404 \pm 0.0067$$

TABLE VII

pH dependence of the dissociation of Oxy-Hr in
0.01 μ Tris-cacodylate buffer and at 25°

pH	C _o (μ g/ml)	α	\bar{M}_w	K _{eq}	ΔG°
10.0	30	0.895	35,400	8.38×10^{-18}	5.825*
9.70	30	0.884	36,300	7.20×10^{-18}	5.847*
9.40	30	0.784	44,300	2.39×10^{-18}	6.010*
9.18	30	0.643	47,600	1.62×10^{-18}	6.068*
9.00	30	0.629	56,700	5.77×10^{-19}	6.221*
8.80	30	-----	62,000	-----	-----
8.57	30	-----	61,200	-----	-----
8.42	30	-----	61,400	-----	-----
8.04	30	-----	62,700	-----	-----
7.81	30	0.472	62,900	1.24×10^{-42}	7.147 [#]
7.60	30	0.477	62,400	1.37×10^{-42}	7.140 [#]
7.40	30	0.482	61,900	1.51×10^{-42}	7.133 [#]
7.20	30	0.487	61,500	1.64×10^{-42}	7.029 [#]
7.00	30	0.478	62,300	1.39×10^{-42}	7.139 [#]
6.60	30	0.477	62,400	1.37×10^{-42}	7.140 [#]
6.00	30	0.535	56,992	4.85×10^{-42}	7.042 [#]
5.80	30	0.560	54,654	5.87×10^{-42}	7.032 [#]
5.70	30	0.651	46,100	2.48×10^{-41}	6.915 [#]
5.60	30	0.701	41,500	5.18×10^{-41}	6.870 [#]
5.40	30	0.910	21,900	1.40×10^{-39}	6.626 [#]
5.30	185	0.276	-----	4.11×10^{-39}	6.546 [#]
5.20	180	0.467	-----	3.17×10^{-37}	6.225 [#]
5.00	30	1.000	13,500	-----	-----
5.00	180	0.515	-----	7.63×10^{-37}	6.160 [#]
4.90	180	0.545	-----	1.28×10^{-36}	6.122 [#]
4.80	180	0.560	-----	1.64×10^{-36}	6.103 [#]

* in Kcal/mole of dimer; # in Kcal/ mole of monomer

TABLE VIII

pH dependence of the dissociation of Oxy-Hr in 0.01 μ Tris-cacodylate at 5°

pH	C _o (μ g/ml)	α	\bar{M}_w	K _{eq}	ΔG°
9.83	30	0.7138	49,900	1.24×10^{-18}	5.698*
9.69	30	0.6875	52,000	9.85×10^{-19}	5.730*
9.35	30	0.6025	58,800	4.56×10^{-19}	5.836*
9.04	30	0.5088	66,300	1.87×10^{-19}	5.960*
8.20	30	-----	70,900	-----	-----
8.00	30	-----	71,500	-----	-----
7.20	30	0.4064	69,000	3.35×10^{-43}	6.758#
7.00	30	0.4107	68,600	3.67×10^{-43}	6.752#
6.64	30	0.4150	68,200	4.02×10^{-43}	6.745#
6.36	30	0.4534	64,600	8.74×10^{-43}	6.692#
5.96	30	0.4626	61,200	1.03×10^{-42}	6.680#
5.70	30	0.5294	57,500	3.51×10^{-42}	6.596#
5.46	30	0.7144	40,200	6.36×10^{-41}	6.396#
5.31	30	0.7882	33,300	1.88×10^{-40}	6.321#
5.10	30	0.9326	19,800	2.27×10^{-39}	6.149#
5.00	30	1.0000	13,500	-----	-----
5.00	100	0.5460	-----	2.03×10^{-38}	5.997#
4.90	100	0.5588	-----	2.60×10^{-38}	5.979#
4.80	100	0.5710	-----	3.20×10^{-38}	5.966#

* in Kcal/mole of dimer ; # in Kcal/mole of monomer

TABLE IX

pH dependence of the dissociation of Oxy-Hr
in 0.01 μ Tris-cacodylate and at 30°

pH	C _o (μ g/ml)	α	\bar{M}_w	K _{eq}	ΔG°
9.60	30	0.926	32,900	1.37×10^{-17}	5.849*
9.40	30	0.919	34,000	1.20×10^{-17}	5.868*
9.28	30	0.889	35,900	7.69×10^{-18}	5.935*
9.04	30	0.711	50,150	1.21×10^{-18}	6.214*
8.80	30	-----	56,100	-----	-----
8.20	30	-----	56,700	-----	-----
7.40	30	0.532	57,300	3.64×10^{-42}	7.186#
7.20	30	0.535	57,000	3.84×10^{-42}	7.182#
7.00	30	0.526	57,800	3.32×10^{-42}	7.193#
6.41	30	0.558	54,800	5.71×10^{-42}	7.152#
6.00	30	0.607	50,226	1.26×10^{-41}	7.092#
5.70	30	0.717	40,000	6.56×10^{-41}	6.968#
5.50	30	0.807	31,545	2.49×10^{-40}	6.868#
5.19	30	1.000	13,500	-----	-----
5.19	180	0.487	-----	3.69×10^{-37}	6.318#
5.00	180	0.535	-----	9.09×10^{-37}	6.250#
4.90	180	0.528	-----	1.10×10^{-36}	6.236#

* in Kcal/molr of dimer ; # in Kcal/mole monomer

The slope of the curve in Figure 19a gives the number of groups (n) being titrated (Edsall and Wyman, 1958, pp. 487-504). The value of n was very close to 8 for the reaction at 25° as shown in Table X. Secondly, according to the procedure of Dixon (Dixon and Webb, 1964, pp. 116-143 and Cassman and Schachman, 1971) two apparent pK values can be obtained from the data in Figure 19a. The apparent pK values were found to be 5.08 and 5.80, respectively. Since more monomer was formed with increasing proton concentration, the proton must bind to the monomer preferentially to octamer. Thus, the apparent pK's were 5.82 for monomer and 5.08 for octamer. An empirical equation (25) was derived which gave a good fit to the experimental data between pH 4.8 and 7.4,

$$(25) \quad K_{ob} = \frac{K_o}{1 + [H^+]^n / [K_a]^n} + \frac{K_H}{1 + [K_a]^n / [H^+]^n}$$

where K_{ob} = dissociation constant of OxyHr at a given pH

K_o = dissociation constant of OxyHr at neutral pH

K_H = dissociation constant of OxyHr at acidic pH

n = number of protons involved in the reaction

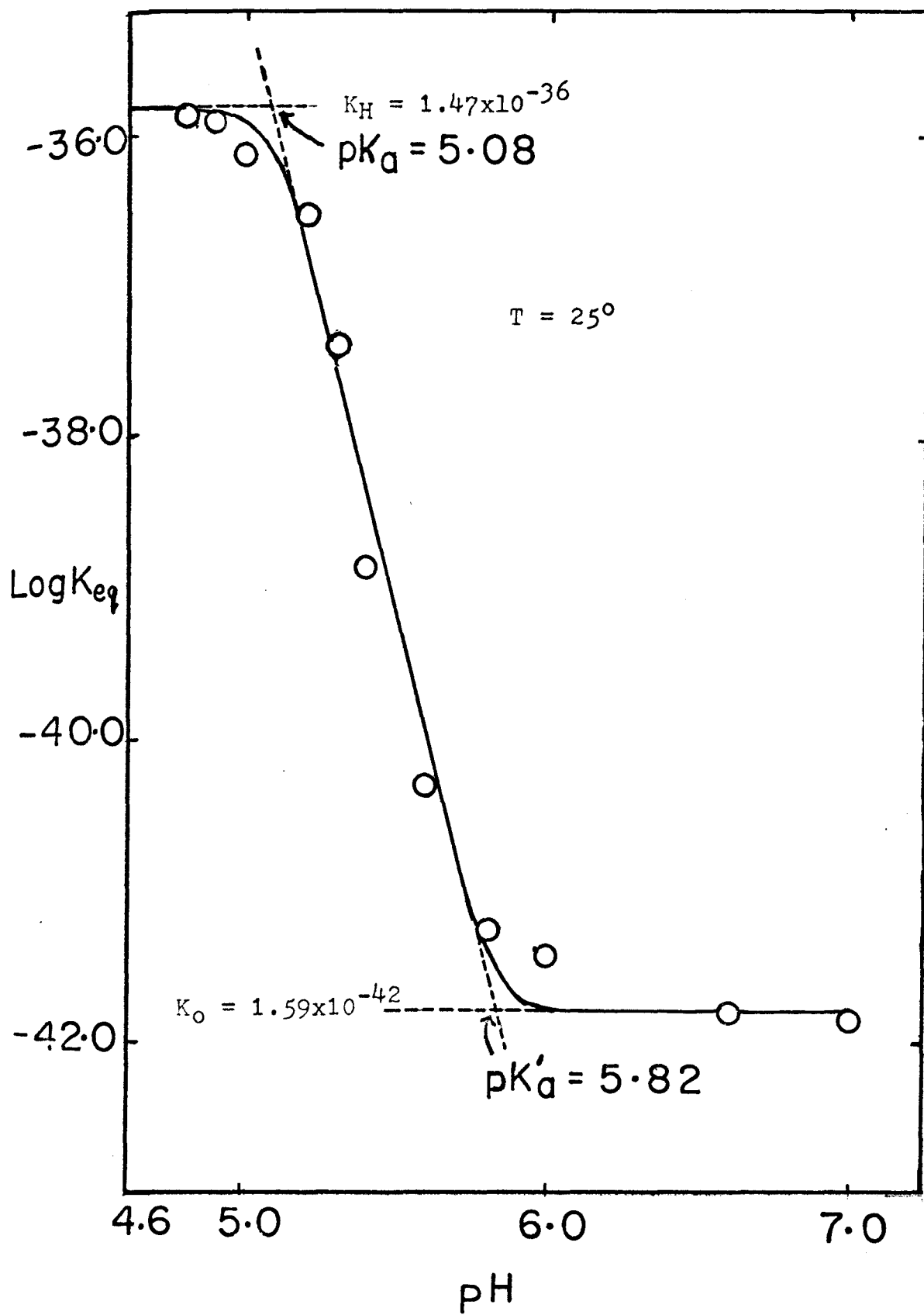
K_a = dissociation constant of the specific amino acid residue involved in the subunit contact in octamer

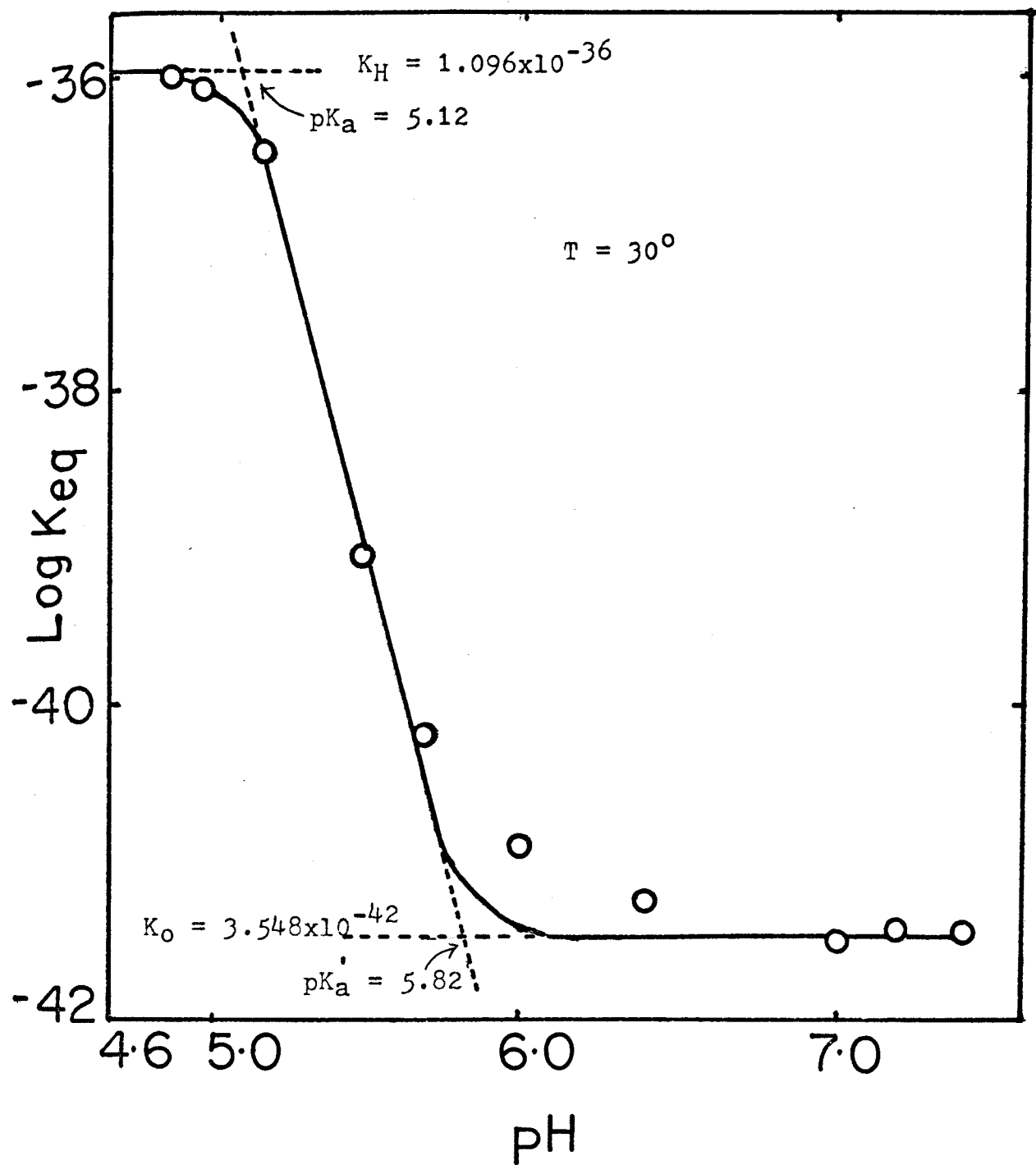
TABLE X

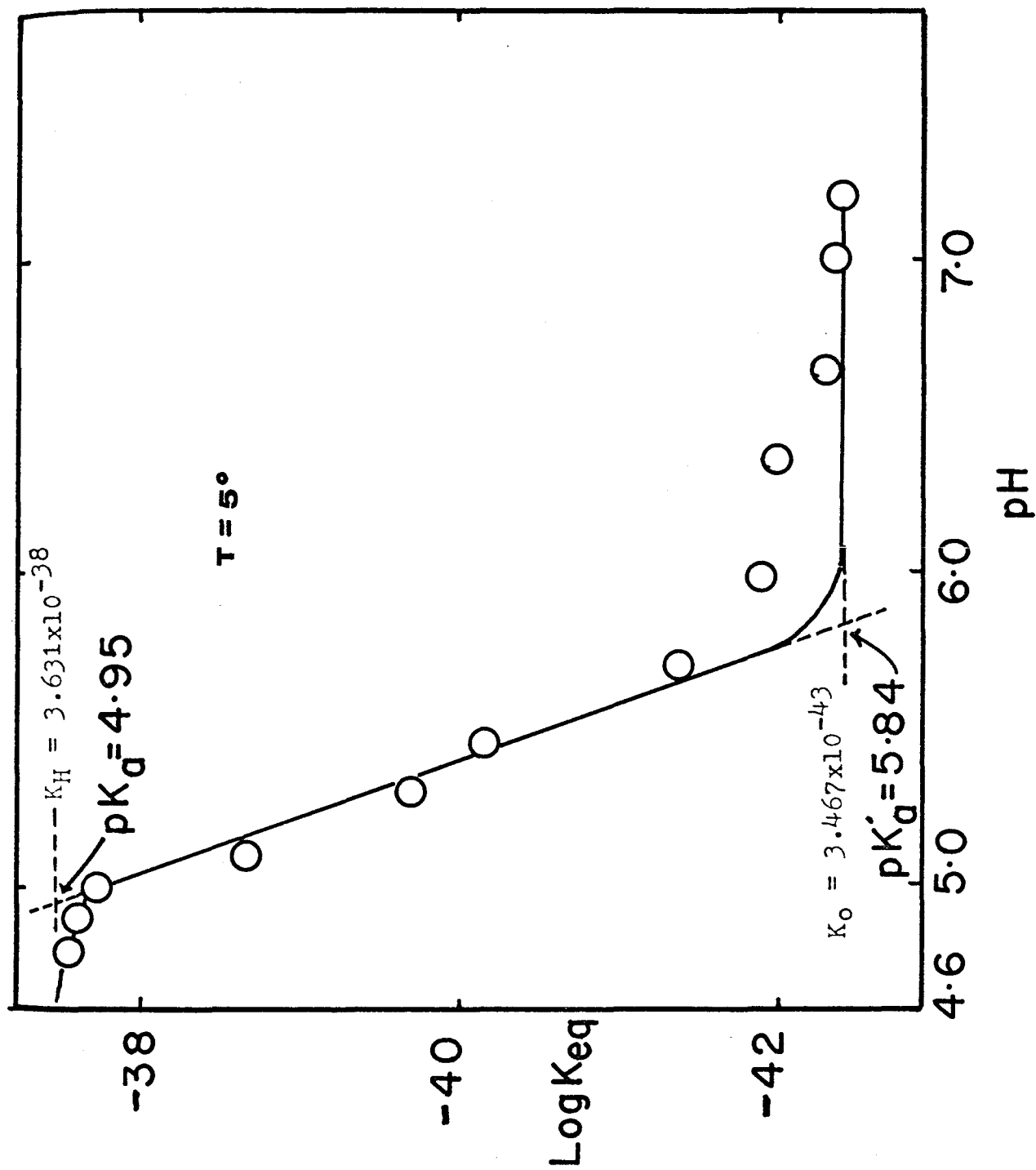
Variation of log K with pH calculated according to equation (25)

T = 5°			T = 25°			T = 30°		
pH	-logK _{ob} [*] (calc)	-logK _{ob} (exp)	pH	-logK _{ob} [*] (calc)	-logK _{ob} (exp)	pH	-logK _{ob} [*] (calc)	-logK _{ob} (exp)
4.60	37.444	-----	4.00	35.832	-----	4.60	35.960	-----
4.80	37.491	37.495	4.80	35.835	35.860	4.90	35.968	35.958
4.90	37.616	37.585	4.90	35.847	35.000	5.00	36.005	36.041
5.00	37.917	37.692	5.00	35.920	36.110	5.19	36.626	36.433
5.10	38.392	38.644	5.20	36.832	36.498	5.50	38.995	39.604
5.31	39.602	39.725	5.30	39.594	37.369	5.70	40.554	40.183
5.46	40.495	40.197	5.40	38.354	38.853	6.00	41.432	40.900
5.70	41.824	41.455	5.60	39.988	40.280	6.41	41.450	41.243
5.96	42.423	41.989	5.80	41.381	41.231	7.00	41.450	41.479
6.36	42.460	42.058	6.00	41.786	41.440	7.20	41.450	41.416
6.64	42.460	42.396	7.00	41.800	41.840	7.40	41.450	41.439
7.00	42.460	42.435						
7.20	42.460	42.475						
* with n=6			*with n=8			*with n = 8		
K _o = 3.47x10 ⁻⁴³			K _o = 1.59x10 ⁻⁴²			K _o = 3.55x10 ⁻⁴²		
K _H = 3.63x10 ⁻³⁸			K _H = 1.47x10 ⁻³⁶			K _H = 1.10x10 ⁻³⁶		
K _a = 1.12x10 ⁻⁵			K _a = 8.43x10 ⁻⁶			K _a = 7.59x10 ⁻⁶		

Figure 19. Dependence of the logarithm of the dissociation constants on pH. Conditions: 0.01 μ Tris-cacodylate buffer. Circles, experimental points; solid line, theoretical curve calculated using equation (25) and the data in Table X. (a) $T = 25^{\circ}$; (b) 30° and (c) 5° .







K'_a = dissociation constant of the same specific amino acid residue in monomer.

The relationship between K_a and K'_a is given in Eq. (26),

$$K_o(K_a)^n = K_H (K'_a)^n \quad (26)$$

The solid curve in Figure 19a was generated by Eq. (25) with the values of $K_o = 1.59 \times 10^{-42}$, $K_H = 1.47 \times 10^{-36}$, $K_a = 8.43 \times 10^{-6}$ and $n = 8$. This calculated curve fits the experimental data at 25° (open circles) reasonably well. The same procedure was also tried for the reaction at 30° and 5° . The calculated values of $\log K_{ob}$ along with the experimentally determined values are shown in Figures 19a, b and c and also in Table X. It should be noted that Eq.(25) fits the experimental data better when n was set equal to 6 rather than 8 for the reaction at 5° . The values of pK and pK'_a at three temperatures were also indicated in Figures 19a, b and c.

Association-Dissociation Reaction between pH 6.6 and 7.4 at 5° , 25° and 30°

Earlier studies on the association-dissociation equilibrium of hemerythrin by sedimentation experiments indicated the predominant interacting species were octamer and monomer at neutral pH (Keresztes-Nagy et al, 1965; Klapper et al, 1968; Langerman et al, 1969). The results obtained in this work

tend to confirm these earlier findings since the values of n have been shown to be close to 8. The procedure for the estimation of n from the analysis of trailing boundary has been described in Chapter I. Table XI shows values of n obtained from the differential elution diagrams.

With the knowledge of n and α the equilibrium constants were calculated according to Eq. (27)

$$K_{eq} = \frac{\alpha^n C_0^{n-1}}{(1 - \alpha)} \quad (27)$$

The values of K_{eq} at three temperatures are listed in Tables VII -IX, respectively.

Association-Dissociation Reaction above pH 9.0 at 5°, 25° and 30°

As in the neutral pH region, the trailing boundary of the elution curve shows bimodality (Figures 12a and b), indicating that n is greater than 2. Using the values of V'_{min} determined from the trailing boundary, σ_l from Table IV and the assumption that only two species were in equilibrium, n was found to be approximately 3.7 ± 0.4 . It is apparent from the data of weight average molecular weight versus pH presented in Figure 11 (also Tables VII-IX) that the two species present in equilibrium above pH 9.0 are octamer and dimer. With the assumption

TABLE XI

Estimation of the stoichiometry of the dissociation, n , from the analysis of the trailing boundaries with the aids of the Eqs. (21) and (22) and also the data of Tables IV and III.

Expt.	pH	$C_o(\mu\text{g/ml})$	$T\text{ }^\circ\text{C}$	$V_o(\text{ml})$	$V_i(\text{ml})$	$S(\text{ml})$	V'_{\min}	v'_m	n
112	9.00	30	25	21.00	42.88	32.0	64.80	0.275	3.60
156	9.04	30	30	22.00	36.42	32.0	-----	-----	-----
113	9.20	30	25	21.76	40.80	34.0	67.00	0.275	3.60
150	9.28	30	30	22.00	36.42	28.0	60.02	0.274	3.67
155	9.39	30	30	22.88	40.72	28.0	61.86	0.269	4.08
118	9.55	30	25	21.76	40.80	32.0	64.80	0.271	3.90
117	9.53	30	25	22.00	36.42	28.0	60.04	0.275	3.60
153	9.80	30	25	21.00	42.88	32.4	65.10	0.273	3.74
121	9.83	30	5	22.00	36.42	28.0	60.20	0.269	4.08
119	7.00	14	25	21.76	40.80	32.0	68.36	0.358	7.77
120	7.00	20.9	25	21.76	40.80	32.0	68.50	0.361	6.96
122	7.00	20	25	21.76	40.80	32.0	68.50	0.361	6.96

of an octamer-dimer equilibrium, equilibrium constants were calculated using Eq. (27). The results are given in Tables VII - IX.

Association-Dissociation Reaction between pH 8.0 and 8.8

Two procedures were used to examine the stoichiometry of the association-dissociation reaction between pH 8.0 and 8.8. The first involved the analysis of the trailing boundary of the elution profile. As indicated previously, since the trailing boundary of the elution profile was unimodal (a single peak in the differential profile), n must equal 2. This is shown in Figure 14. The second method of evaluating n is based on the relationship represented by Eq. (28)

$$\log (1 - \alpha_1)C_0 = n \log (\alpha_1 C_0) - \log K_{eq} \quad (28)$$

where C_0 is the protein concentration and α_1 is the weight fraction of monomer. A plot of $\log (1 - \alpha_1)C_0$ versus $\log (\alpha_1 C_0)$ should yield a straight line with a slope of n. α_1 may in turn be evaluated from a study of the dependence of the weight average molecular weight on protein concentration. Equation (29) describes the relationship between α_1 , \bar{M}_w and C (Steiner, 1952)

$$\ln \alpha_1 = \int_0^{C_0} \left\{ \left[\frac{M_1}{\bar{M}_w} - 1 \right] / C \right\} dC \quad (29)$$

A series of elution profiles were obtained at different protein concentrations. These are shown in Figure 20. From these data the weight average molecular weights, \bar{M}_w , were evaluated. These are tabulated in Table XII. A plot was then made of \bar{M}_w against C_0 (Figure 21). Data pairs of \bar{M}_w and C_0 were taken from this curve and used to numerically evaluate the term $(M_1 / \bar{M}_w - 1) / C$ from Eq. (29). $(M_1 / \bar{M}_w - 1) / C$ was then plotted against C_0 (Figure 22). Values of α_1 at different protein concentrations, C_0 , were then obtained by integrating the area under the curve. Integral values were approximated using the trapezoidal rule. The values of α_1 and C_0 are listed in Table XIII and are graphed according to Eq. (28) in Figure 23. The slope of the line in Figure 23 was found to be close to 2.

The stoichiometry $n = 2$ suggests that the reaction is a dimerization. It does not, however, mean that the reaction is a simple dimerization between two species, since consecutive dimerization (Cassman and Schachman, 1971) and continuous polymerization (Chianocce et al, 1968; Cassmann and Schachman, 1971) could also give rise to unimodality in the sedimentation pattern or trailing boundary of the elution profile. It may be possible to distinguish between some of these models by examining the weight average molecular weight as a function of protein concentration.

Figure 20. Dependence of the dissociation of oxyhemerythrin on protein concentration at pH 8.04 and 25°. Absorbance was monitored either at 220 nm or 280 nm.

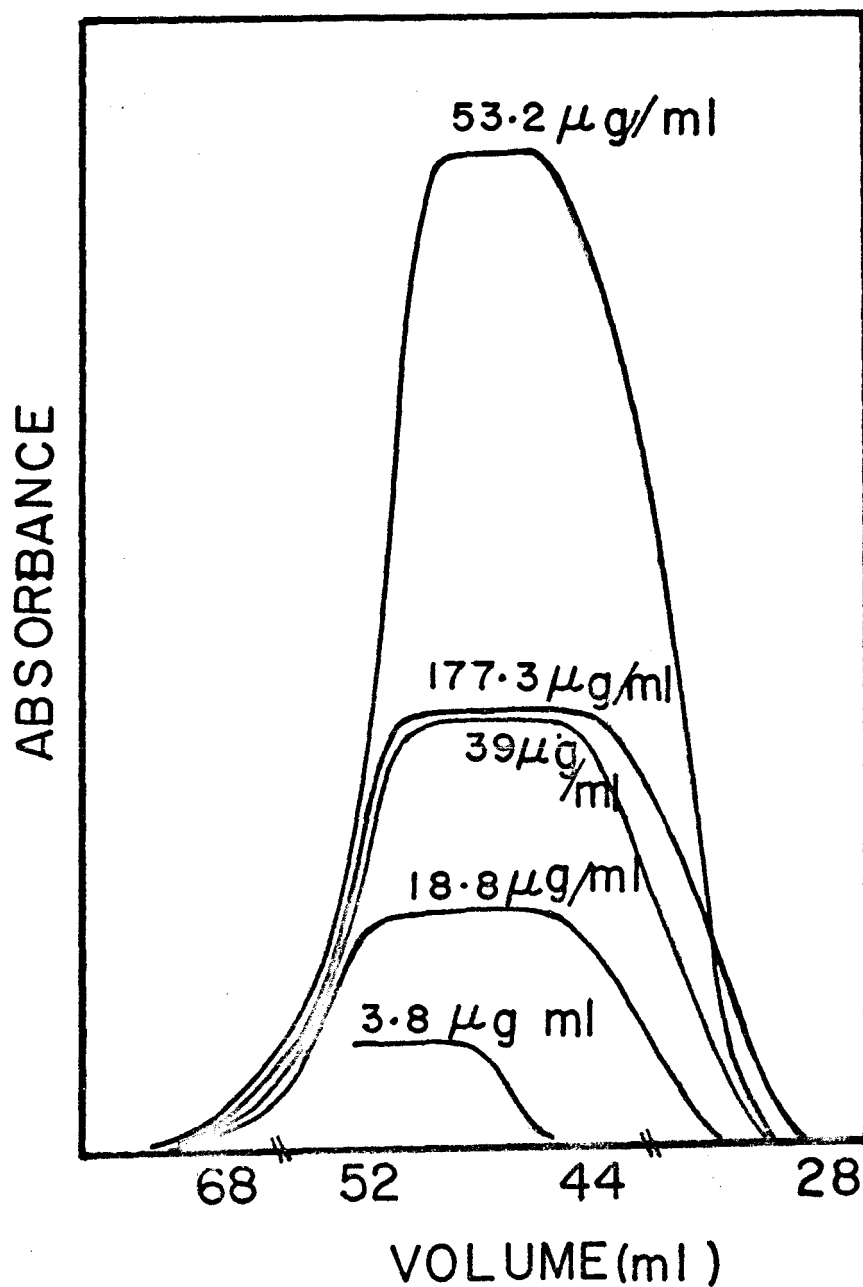


Figure 21. Dependence of the molecular weight of oxyhemerythrin upon protein concentration at pH 8.04 and 25°. The conditions were given in Figure 20.

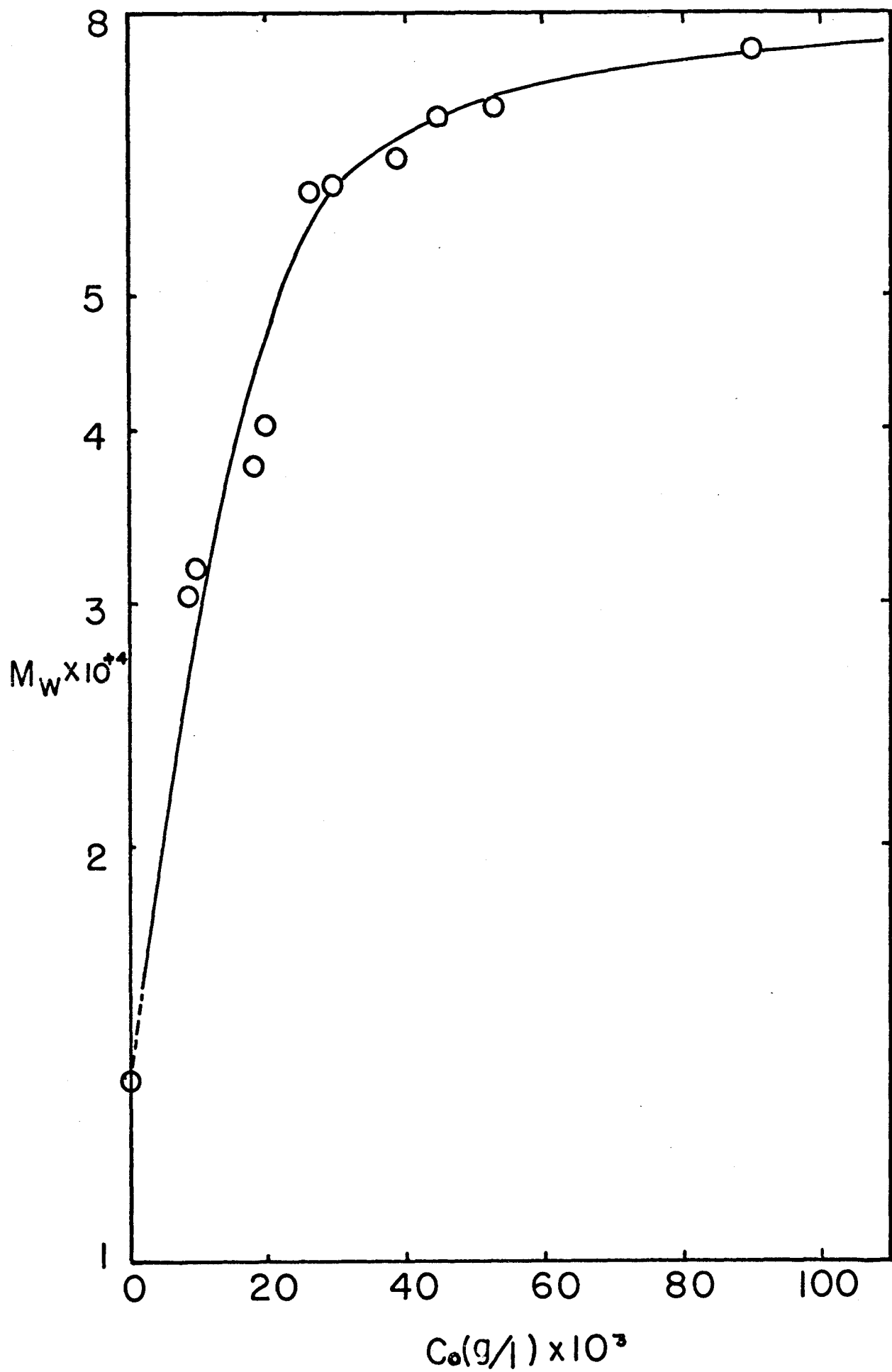


TABLE XII

Molecular weight data on the simple dimerization between 'monomer' and 'dimer' of oxyhemerythrin in 0.01 μ pH 8.04 Tris-cac and 25°

$C_o(g/l)$	\bar{M}_w	$(\bar{M}_w - M_1)$	$(2M_1 - \bar{M}_w)^2$	$\frac{(\bar{M}_w - M_1)}{(2M_1 - \bar{M}_w)^2}$	$\frac{(\bar{M}_w - M_2)}{(2M_2 - \bar{M}_w)^2}$	$\frac{(\bar{M}_w - M_4)}{(2M_4 - \bar{M}_w)^2}$
0.1773	79,900	66,400	27.98×10^8	0.237×10^{-4}	0.759×10^{-4}	0.359×10^{-4}
0.0900	76,500	63,000	24.50×10^8	0.257×10^{-4}	0.936×10^{-4}	0.247×10^{-4}
0.0532	68,000	54,500	16.81×10^8	0.324×10^{-4}	1.949×10^{-4}	0.095×10^{-4}
0.0450	67,100	53,600	16.08×10^8	0.333×10^{-4}	2.167×10^{-4}	0.085×10^{-4}
0.0390	62,900	49,400	12.89×10^8	0.384×10^{-4}	4.063×10^{-4}	0.048×10^{-4}
0.0295	60,300	46,800	11.09×10^8	0.422×10^{-4}	7.201×10^{-4}	0.031×10^{-4}
0.0266	59,500	46,000	10.56×10^8	0.436×10^{-4}	9.028×10^{-4}	0.027×10^{-4}
0.0200	40,300	26,800	1.88×10^8	1.515×10^{-4}	0.763×10^{-4}	-0.030×10^{-4}
0.0188	37,400	23,900	1.08×10^8	2.209×10^{-4}	0.402×10^{-4}	-0.032×10^{-4}
0.0100	31,800	18,370	0.24×10^8	7.745×10^{-4}	0.104×10^{-4}	-0.038×10^{-4}
0.0088	30,300	16,800	0.11×10^8	15.427×10^{-4}	0.061×10^{-4}	-0.039×10^{-4}
0.0038	16,000	2,500	1.21×10^8	0.027×10^{-4}	-0.079×10^{-4}	-0.045×10^{-4}

Figure 22. Estimation of weight fraction of monomer, α , ,
by graphical integration according to equation (29).
Conditions were given in Table XII.

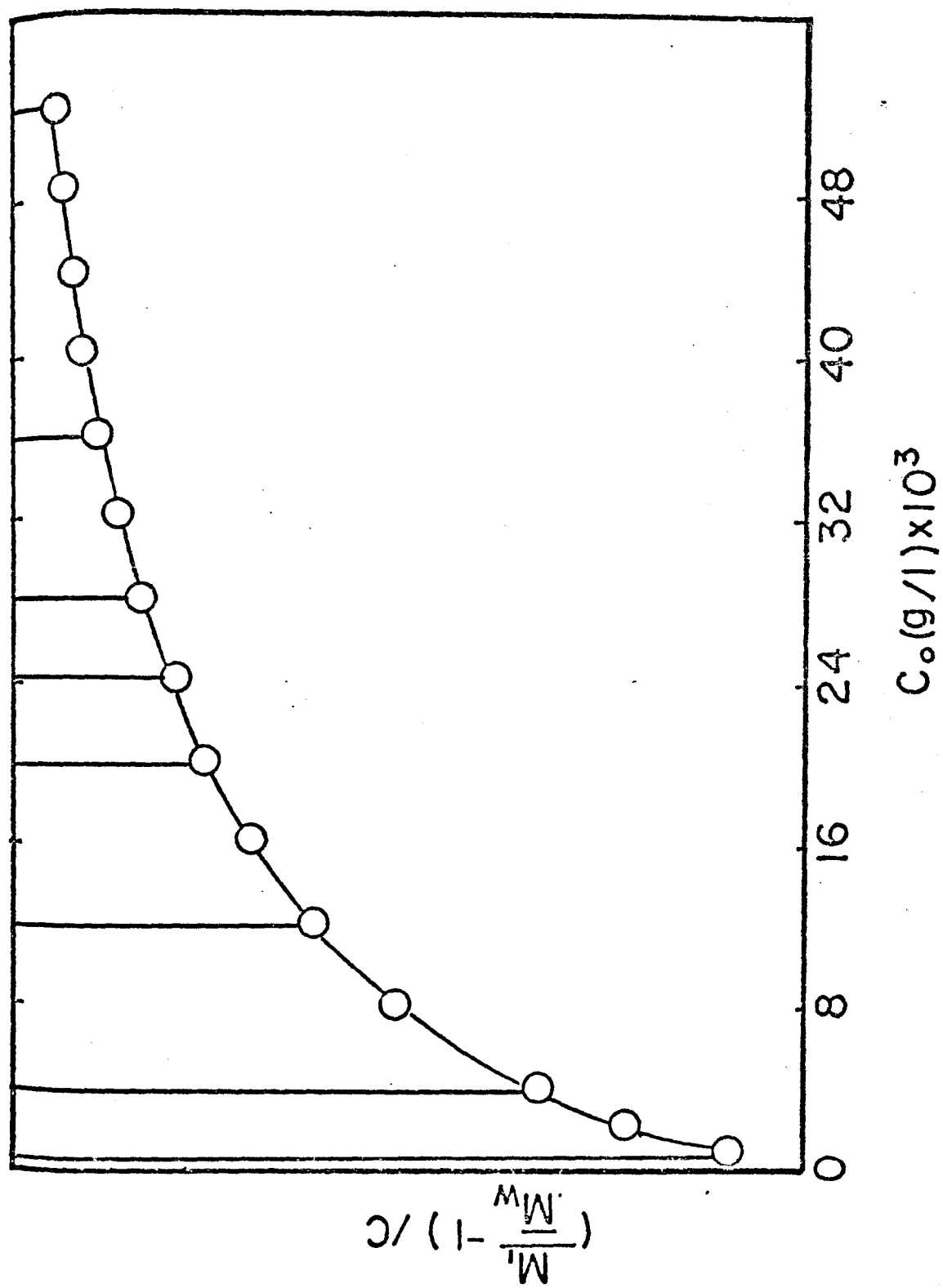
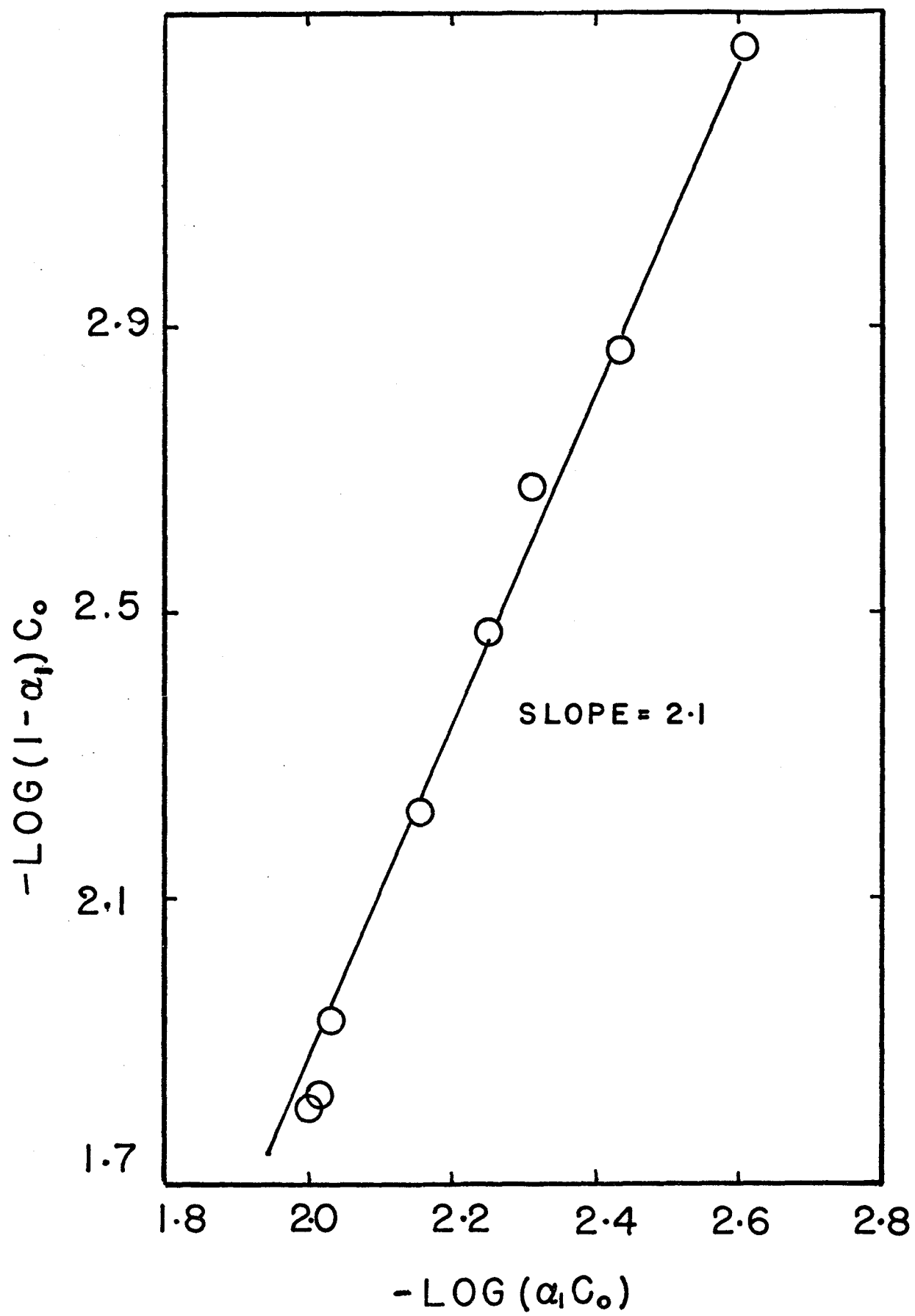


TABLE XIII

Variation of weight fraction of monomer of oxyhemerythrin at different protein concentrations, pH 8.04 and 25°

C_o (g/l)	α_1	$(1 - \alpha_1)$	$-\log(\alpha_1 C_o)$	$-\log(1 - \alpha_1) C_o$
0.003	0.829	0.1710	2.604	3.290
0.005	0.7303	0.2697	2.483	2.870
0.007	0.7004	0.2997	2.309	2.678
0.009	0.6223	0.3777	2.252	2.468
0.013	0.5386	0.4614	2.155	2.222
0.021	0.4439	0.5564	2.031	1.933
0.025	0.3750	0.6250	2.028	1.806
0.029	0.3371	0.6629	2.010	1.716
0.037	0.2820	0.7180	1.980	1.576

Figure 23. Estimation of the stoichiometry of the association-dissociation reaction of oxyhemerythrin at pH 8.04 and 25° according to equation (28).



Let us begin by considering the simple dimerization model. If one assumes a monomer \rightleftharpoons dimer equilibrium and plots $(\bar{M}_w - M_1)/(2M_1 - \bar{M}_w)^2$ versus C_0 (Appendix 1), the results are shown in Table XII and Figure 24. The plot shows a pronounced upwark curvature beyond 5 $\mu\text{g/ml}$ and when this is combined with the observation that experimentally determined \bar{M}_w beyond this protein concentration are greater than dimer (M.W. = 27,000), it becomes clear that polymerization proceeded beyond the dimer stage. Similarly, if one assumes a dimer \rightleftharpoons tetramer equilibrium, and again plots $(\bar{M}_w - M_2)/(2M_2 - \bar{M}_w)^2$ versus C_0 , the results are shown in Figure 25 and Table XII. The plot shows an abrupt upward curvature at concentration greater than 20 $\mu\text{g/ml}$. Finally, if one assumes a tetramer \rightleftharpoons octamer equilibrium by plotting $(\bar{M}_w - M_4)/(2M_4 - \bar{M}_w)^2$ versus C_0 (Figure 26 and Table XII), the curve is also nonlinear. Moreover, the \bar{M}_w at the lowest protein concentration is too low to be consistent with a tetramer-octamer equilibrium. These results clearly indicate that the interacting system contains more than two species. Hence, the association-dissociation reaction between pH 8.0 and 8.8 is either a consecutive dimerization or continuous polymerization. For the consecutive dimerization model monomer \rightleftharpoons dimer \rightleftharpoons tetramer \rightleftharpoons octamer, the relevant equations were derived and are presented in the Appendix 2. These equations are

Figure 24. Plot of $(\bar{M}_w - M_1) / (2M_1 - \bar{M}_w)^2$ versus C_o .
Conditions were given in Table XII.

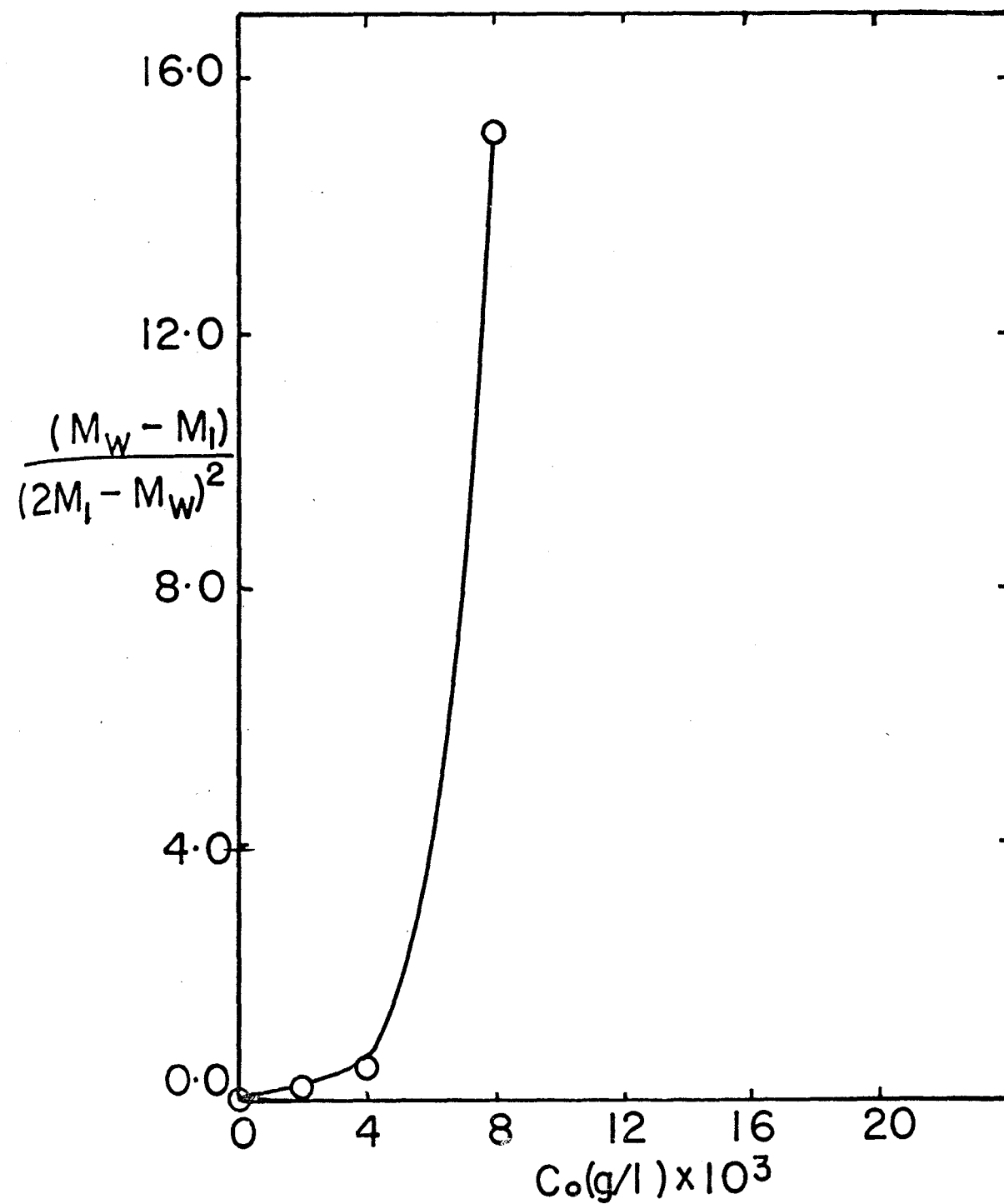


Figure 25. Plot of $(\bar{M}_w - M_2) / (2M_2 - \bar{M}_w)^2$ versus C_o .
Conditions: see Table XII.

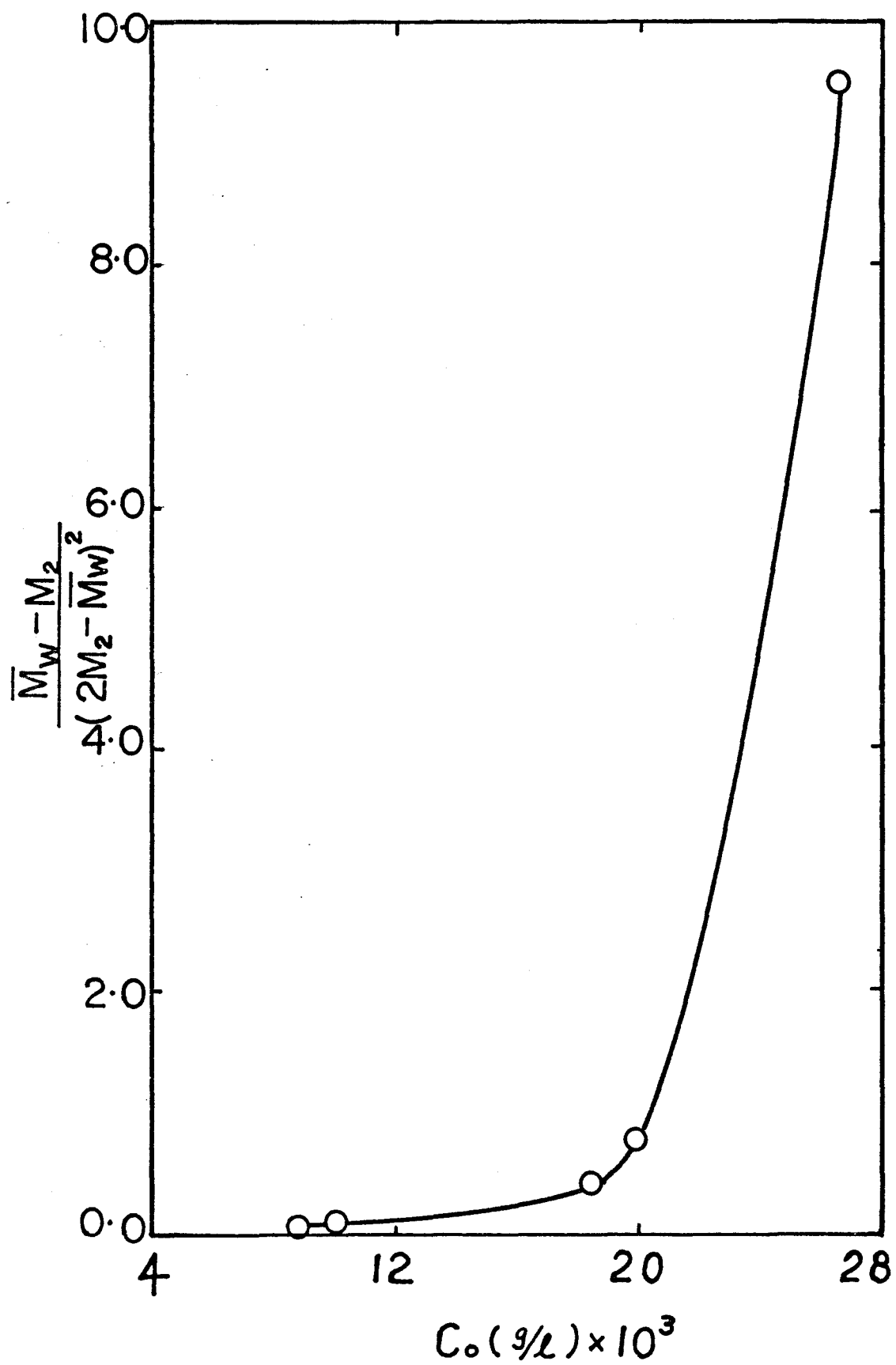
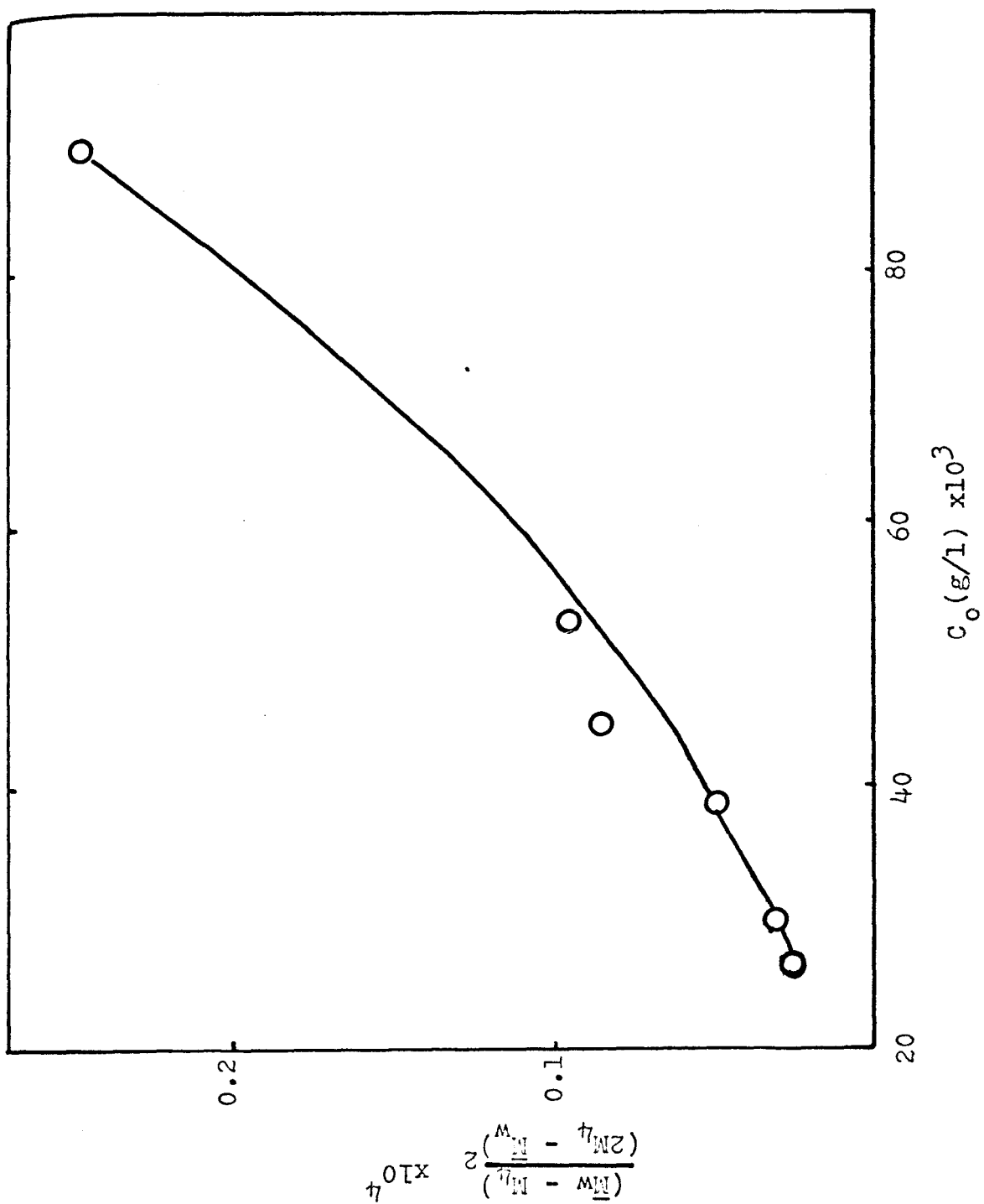


Figure 26. Plot of $(\bar{M}_w - M_4) / (2M_4 - \bar{M}_w)^2$ versus C_o .
Conditions were given in Table XII.



$$K_2 = C_2 / C_1^2 \quad ; \quad C_2 = K_2 (C_1)^2$$

$$K_4 = C_4 / C_2^2 \quad ; \quad C_4 = K_4 (C_2)^2 = K_4 K_2^2 (C_1)^4 = K'_4 C_1^4$$

$$K_8 = C_8 / C_4^2 \quad ; \quad C_8 = K_8 (C_4)^2 = K_8 K_4^2 K_2^4 C_1^8 = K'_8 C_1^8$$

Since the total concentration C_0 is the sum of all the species present in the mixture, we get

$$C_0 = C_1 + C_2 + C_4 + C_8 = \sum_i C_i = \sum_i K_i C_1^i \quad (30)$$

where C_i = concentration of i -species in gm/liter. Let

α_1 = weight fraction of monomer, then $C_1 = \alpha_1 C_0$. Expressing C_i in terms of C_0 in Eq. (30), we obtain

$$C_0 = \alpha_1 C_0 + K_2 (\alpha_1 C_0)^2 + K'_4 (\alpha_1 C_0)^4 + K'_8 (\alpha_1 C_0)^8 \quad (31)$$

$$\text{or } C_0 (1 - \alpha_1) = K_2 (\alpha_1 C_0)^2 + K'_4 (\alpha_1 C_0)^4 + K'_8 (\alpha_1 C_0)^8 \quad (32)$$

$$\text{where } K'_4 = K_4 K_2^2 \quad \text{and} \quad K'_8 = K_8 K_4^2 K_2^4$$

Equation (32) may be solved for K 's if values of α_1 are known at various protein concentrations. Indeed, α_1 as a function of protein concentration has been obtained as shown in Figure 22

and Table XIII. The three equilibrium constants could then be obtained by solving three simultaneous equations at different protein concentrations. The values of these constants are indicated in Table XIV.

TABLE XIV

* Equilibrium constants for the consecutive dimerization of oxyhemerythrin at pH 8.04.				
i	K_i (g/l)	K_i (mole/l)	K'_i (g/l)	K'_i (mole/l)
2	83	5.59×10^5	83	5.59×10^5
4	58.6	1.15×10^2	4.04×10^5	2.51×10^{17}
8	773	6.11×10^8	1.26×10^{14}	1.30×10^{42}

* For definition of constants, see p.93 and the Appendix 2 .

The individual formation constants K_2 , K'_4 and K'_8 together with values for α_i at various protein concentrations, C_o , were used to back calculate the curve of \bar{M}_w^c versus C_o according to Eq. (33) (see Appendix 2)

$$\bar{M}_w^c = \alpha_1 M_1 + 2K_2 C_o \alpha_1^2 M_1 + 4K'_4 C_o^3 \alpha_1^4 M_1 + 8K'_8 C_o^7 \alpha_1^8 M_1 \quad (33)$$

where M_1 = M.W. of monomeric hemerythrin = 13,500. For each protein concentration, C_o , one α_i is obtained by Eq. (29) and the values of \bar{M}_w^c are collected in Table XV.

TABLE XV

Comparison of the theoretical weight average molecular weight (\bar{M}_w^{calc}) and the experimentally determined \bar{M}_w , pH 8.04 and 25°.

$^1C_o(\text{g/l})$	$^1\alpha_1$	$^2\bar{M}_w^{\text{calc}}$	$^3\bar{M}_w$
0.003	0.829	17,000	18,000
0.005	0.730	18,800	20,400
0.007	0.700	19,600	25,000
0.009	0.622	20,000	30,000
0.013	0.539	25,700	34,000
0.021	0.444	49,300	48,000
0.025	0.375	51,700	53,000
0.029	0.337	57,300	60,000
0.037	0.282	68,500	62,000

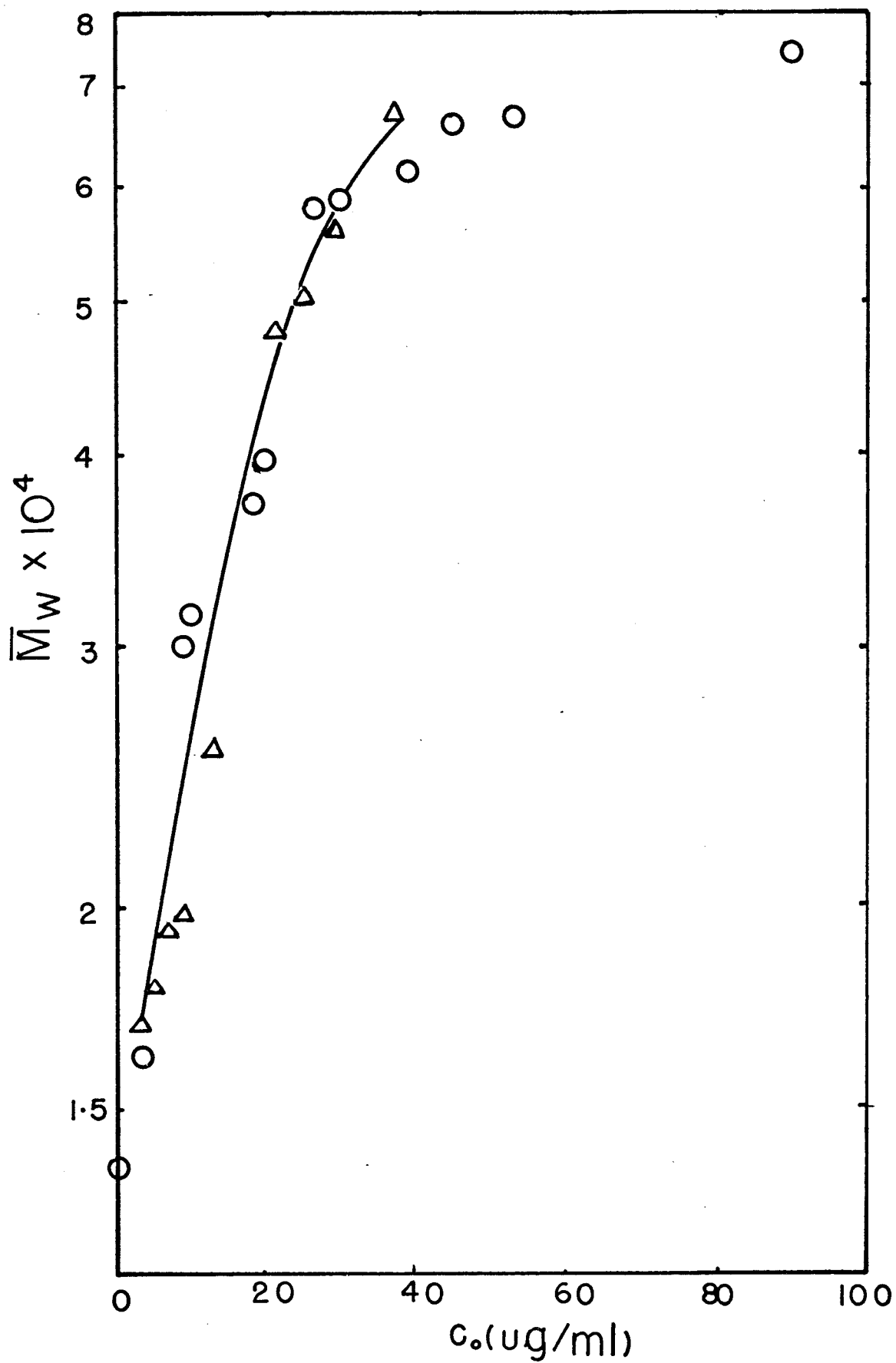
1. Data are from Table XIII.
2. Theoretical \bar{M}_w^{calc} are calculated by Eq.(33).
3. Data obtained from the smooth curve of the experimentally determined \bar{M}_w versus C_o as shown in Figure 21.

Figure 27. Comparison of the weight average molecular weight (\bar{M}_w^{calc}) and the experimentally determined \bar{M}_w as a function of oxyhemerythrin concentration at pH 8.04 and 25°.

Open circles : experimental values of \bar{M}_w ;

Triangles : Values of \bar{M}_w^{calc} calculated by equation (33).

The smooth curve is drawn through the calculated \bar{M}_w^{calc} .



As can be seen, the calculated values show moderate agreement with the experimental curve over a restricted range of protein concentrations. It is also possible to calculate the relative amounts of each subspecies present in the reaction mixture at each protein concentration. Once the weight fraction of monomer, α_1 , is known, the concentration of other species can be estimated from C_0 and the individual formation constant through Eq.(34) :

$$C_1 = \alpha_1 C_0 \dots (34a); \quad C_2 = K_2 (\alpha_1 C_0)^2 \dots (34b)$$

$$C_4 = K_4' (\alpha_1 C_0)^4 \dots (34c); \quad C_8 = K_8' (\alpha_1 C_0)^8 \dots (34d)$$

A graphical display of the relative amounts of each subspecies presented in terms of percentage are plotted against C_0 in Figure 28, and tabulated in Table XVI.

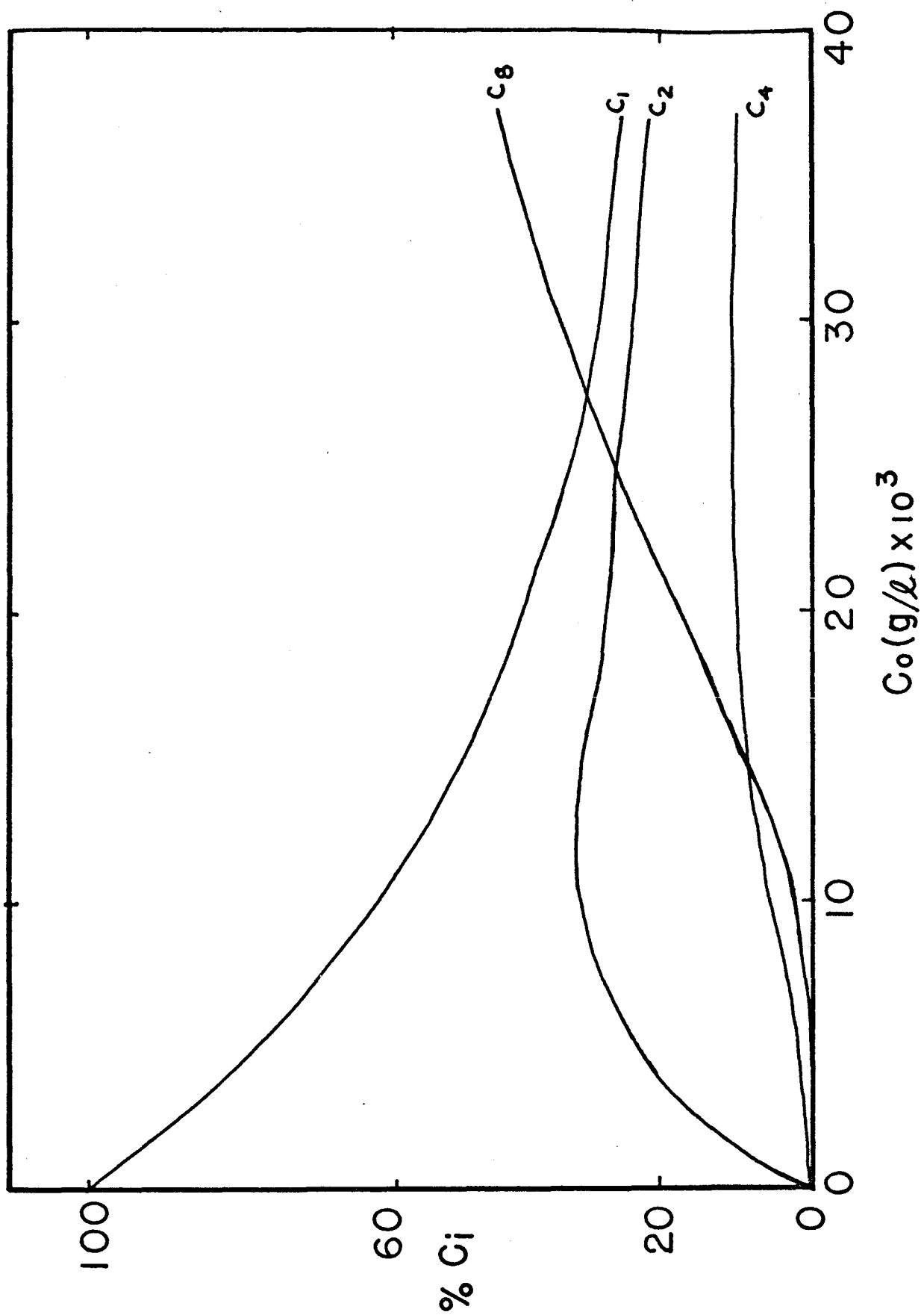
TABLE XVI

Percent of C_i in the reaction mixture at various protein concentrations, pH 8.04 and 25°					
$C_0(g/l) \times 10^{-3}$	α_1	C_1	C_2	C_4	C_8
3.0	0.829	82.3	17.6	0.6	0.01
5.0	0.730	73.0	24.0	1.6	0.11
7.0	0.700	68.8	28.0	3.2	0.59
9.0	0.622	64.2	30.0	4.5	1.37
13.0	0.539	54.8	31.8	7.6	5.70
21.0	0.444	35.0	27.1	11.4	26.80
25.0	0.375	34.4	26.8	11.5	27.60
29.0	0.337	30.7	25.0	11.5	33.00
37.0	0.282	25.0	21.6	11.4	42.10

Another possible model which may describe the experimental results is the continuous polymerization, monomer \rightleftharpoons dimer \rightleftharpoons trimer $\rightleftharpoons \dots \rightleftharpoons$ heptamer \rightleftharpoons octamer. The theoretical equation that describes this type of equilibria was derived by Steiner in 1952. The equation is

$$C_0 \bar{M}_w = \alpha_1 C_0 M_1 + 2K_2 (\alpha_1 C_0)^2 M_1 + \dots + nK_2 \dots K_n (\alpha_1 C_0)^n M_1 \quad (35)$$

Figure 28. The variation in weight fraction, C_i , of monomer (C_1), dimer (C_2), tetramer (C_4), and octamer (C_8), with total protein concentration in 0.01μ Tris-cacodylate buffer, pH 8.04 at 25°



K_2 is the association constant for the formation of dimer from monomer, K_3 is the formation constant of trimer from dimer, etc. This model may be indistinguishable from the consecutive dimerization model (Chiancone et al, 1968; Cassman and Schachman, 1971). This model may give a better fit to data since there are more adjustable constants in Eq. (35) than in Eq. (33). For the reasons which I shall state in the discussion, I did not use my data of \bar{M}_w at different protein concentrations to test for this model of polymerization.

Effects of Temperature on the Association-dissociation of Oxyhemerythrin between pH 4.8 and 7.4

As might be expected, all the qualitative aspects of the association-dissociation equilibrium (i.e. shape of the elution profiles, the rate and stoichiometry of the reaction, and the pH dependence) which were observed at 25°, remained essentially the same at 5° and 30°. The values of K_{eq} and pK_{app} were obtained and are listed in Tables VII, VIII and IX, respectively. The apparent heat of ionization, Q' , of an amino acid residue which participates in the subunit contact, may be estimated from a study of temperature dependence of its pK . With the values for pK_a and pK'_a from Table XVII, $1/T$ was plotted versus pK according to the Wyman equation (1939),

$$Q' = 4.576 \left[\frac{\partial(\text{pK})}{\partial(1/T)} \right] \quad (36)$$

Table XVII

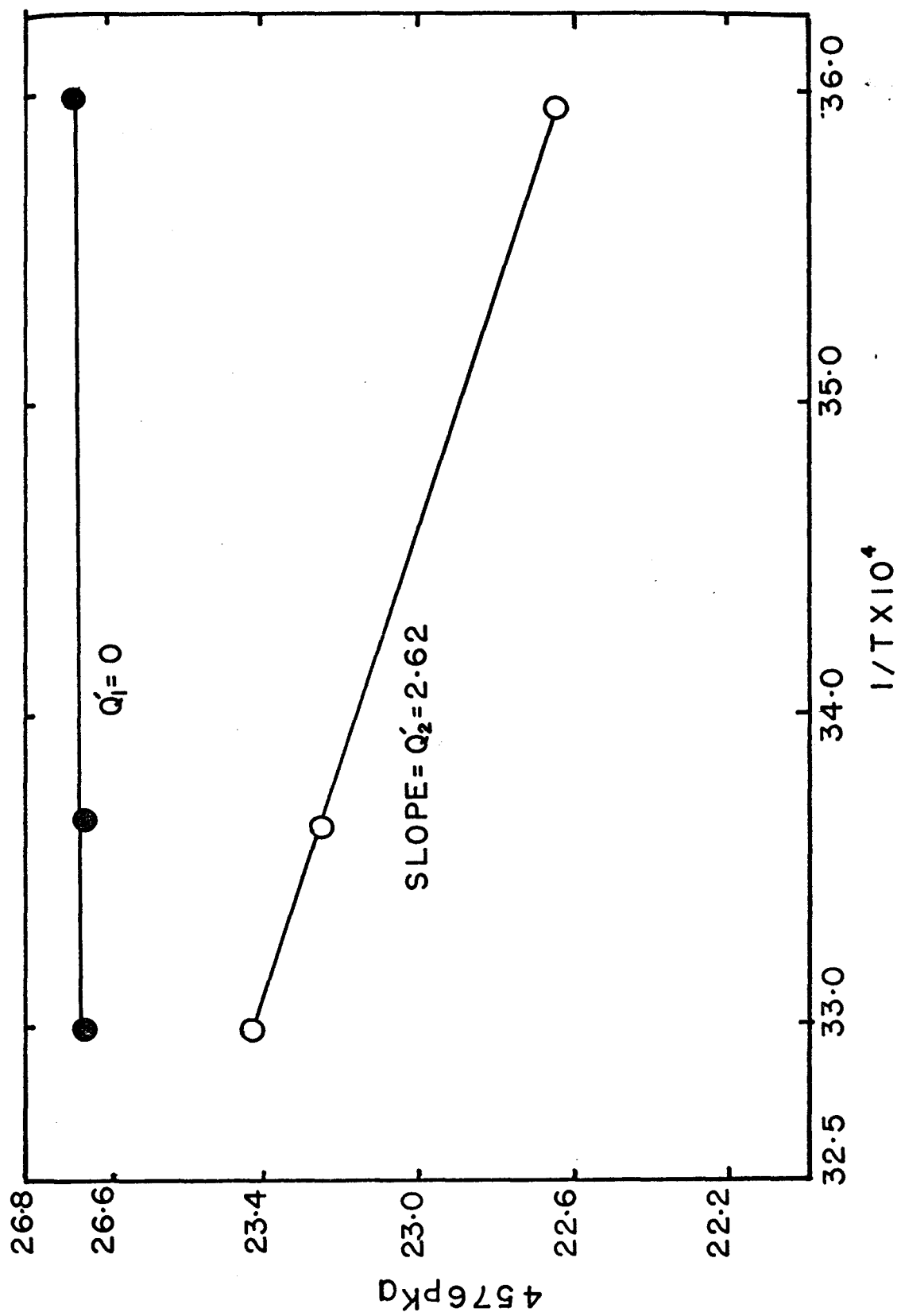
Temperature dependence of the pK_a and pK'_a in 0.01 μ Tris-cacodylate buffer.					
T °K	1/T ($\times 10^{-4}$)	pK_a	4.576pK_a	pK'_a	$4.576\text{pK}'_a$
278.16	35.95	4.95	22.651	5.84	26.724
298.16	33.53	5.08	23.246	5.82	26.632
303.16	32.98	5.12	23.439	5.82	26.632

Figure 29 shows that the slopes were 2.6 and zero for the specific amino acid residues in the octamer and the monomer.

From the knowledge of the temperature dependence of K_{eq} at several pH values , it is possible to calculate the enthalpy changes, ΔH^0 , for the association-dissociation reaction as a function of pH. These calculations were carried out using the integral form of van't Hoff equation,

$$\ln K_2 / K_1 = - \Delta H^0 / R \left[\left(1/T_2 - 1/T_1 \right) \right] \quad (37)$$

Figure 29. Estimation of the apparent heat of ionization (Q') according to equation 36 and the data of Table XVII.



Knowing the equilibrium constants at 25° and 5° , and assuming ΔH° in this temperature range did not change appreciably, one can readily obtain ΔH° . Such data are collected in Table XVIII. In the neutral pH and at 25° , ΔH° was small and positive (about 1.4 ± 1.6 Kcal/ mole of monomer). This value agrees closely with the data obtained from gel filtration studies (Rao and Keresztes-Nagy, 1972b) and also with that from microcalorimetric measurements by Langerman et al (1971). In Figure 30, ΔH° is plotted as a function of pH. The half height of this plot gives the apparent pK of about 5.4 of the amino acid residue involved in the subunit contacts. In addition, the enthalpy of ionization, ΔH_i° , could be evaluated from the difference between ΔH_t° (total enthalpy change) at acidic pH (for example at pH 4.8, the specific side chain being nearly all protonated) and ΔH_d° , the enthalpy change at neutral pH. The ΔH_d° obtained at neutral pH consists only of the enthalpy of dissociation, while ΔH_t° may be considered as the sum of enthalpy change of dissociation (ΔH_d°) and that from the ionization of specific side chain (ΔH_i°). This is illustrated by Eq.(38),

$$\Delta H_t^{\circ} = \Delta H_d^{\circ} + \Delta H_i^{\circ} \quad (38)$$

TABLE XVIII

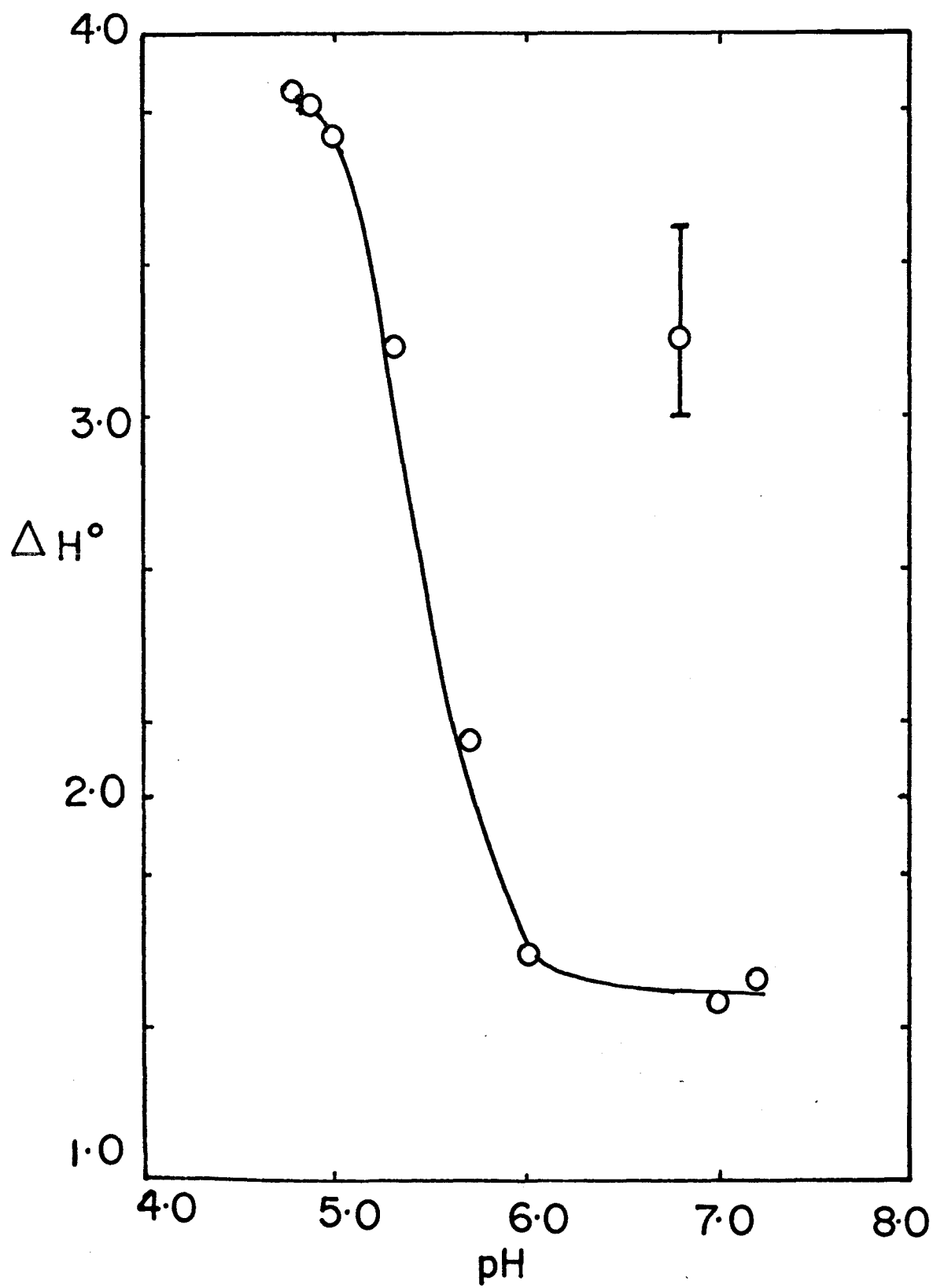
Determination of enthalpy change of the dissociation reaction of pH. The medium was in 0.01 μ Tris-cacodylate

pH	T °C	*K _{eq} xM ⁻⁷	log K _{eq}	ΔH° (Kcal/mole) [#]
4.80	25	1.64x10 ⁻³⁶	-35.87	3.84 \pm 1.62
4.80	5	3.20x10 ⁻³⁸	-37.50	
4.90	25	1.28x10 ⁻³⁶	-35.89	3.81 \pm 1.62
4.90	5	2.60x10 ⁻³⁸	-37.59	
5.00	25	7.63x10 ⁻³⁷	-36.12	3.72 \pm 1.62
5.00	5	2.03x10 ⁻³⁸	-37.69	
5.30	25	4.28x10 ⁻³⁸	-37.37	3.17 \pm 1.62
5.30	5	1.88x10 ⁻⁴⁰	-39.73	
5.70	25	2.48x10 ⁻⁴¹	-40.55	2.15 \pm 1.62
5.70	5	3.51x10 ⁻⁴²	-41.45	
6.00	25	4.85x10 ⁻⁴²	-41.31	1.60 \pm 1.62
6.00	5	1.03x10 ⁻⁴²	-41.99	
6.60	25	1.37x10 ⁻⁴²	-41.86	1.26 \pm 1.62
6.60	5	4.02x10 ⁻⁴³	-42.40	
7.00	25	1.39x10 ⁻⁴²	-41.86	1.46 \pm 1.62
7.00	5	3.35x10 ⁻⁴³	-42.47	
7.20	25	1.64x10 ⁻⁴²	-41.79	1.53 \pm 1.62
7.20	5	3.67x10 ⁻⁴³	-42.43	

* Data from Tables VII, VIII and IX.

The error in ΔH° was estimated according to the Wiberg formula (Wiberg, 1964) and was based on the assumption that the fractional error in the equilibrium constant was 0.1.

Figure 30. Enthalpy of dissociation for oxyhemerythrin as a function of pH. Data were taken from Table XVIII.



From Table XIII, one has ΔH_t^0 (at pH 4.8) = 3.84 Kcal/mole,
and ΔH_d^0 (at pH 7.0) = 1.46 Kcal/ mole, then

$$\Delta H_i^0 = \Delta H_t^0 - \Delta H_d^0 = 2.4 \text{ Kcal/mole.}$$

We have thus obtained the apparent heats of ionization by two procedures. These values of 2.6 (Q') and 2.4 (ΔH_i^0) Kcal/mole of monomer are in good agreement.

Calculation of Other Thermodynamic Parameters at pH 7.00

The association-dissociation equilibrium of oxyhemerythrin at pH 7.00 was further studied in some detail at five different temperatures. The data on the equilibrium constants is given in Table XIX. The corresponding free energy change for the reaction at any given temperature was calculated by Eq. (39),

$$\Delta G^0 = -RT \ln K'_{eq} \quad (39)$$

where K'_{eq} is the association constant of octamer from monomer. Also, the enthalpy change, ΔH^0 , at a given temperature was estimated from the slope of the curve of $d (R \ln K'_{eq})$ versus $1/T$ according to van't Hoff's equation,

$$\frac{d (R \ln K'_{eq})}{d (1/T)} = \Delta H^0 \quad (40)$$

TABLE XIX

Temperature dependence and the thermodynamic parameters
of the association reaction of oxyhemerythrin in 0.01μ
pH 7.00 Tris-cacodylate buffer. $[Oxy-Hr] = 30\mu g/ml$

T °K	$1/T \times 10^{-4}$	\bar{M}_w	$K_{eq} \times M^{-7}$	*Rln K'	$-\Delta G^0$ (Kcal/mole)	ΔH^0 (Kcal/mole)	ΔS^0 (e.u.)
308.16	32.45	60,300	2.07×10^{-42}	190.75	7.35 ± 0.01	-1.32 ± 1.62	21 ± 5
303.16	32.98	61,800	1.59×10^{-42}	191.37	7.25 ± 0.01	-1.32 ± 1.62	21 ± 5
298.16	33.59	62,100	1.45×10^{-42}	191.45	7.14 ± 0.01	-1.32 ± 1.62	21 ± 6
298.16	33.59	63,000	1.21×10^{-42}	191.82	7.15 ± 0.01	-1.32 ± 1.62	21 ± 6
293.16	34.11	64,000	9.89×10^{-43}	192.22	7.04 ± 0.01	-1.32 ± 1.62	20 ± 6
283.16	35.31	68,000	4.20×10^{-43}	193.92	6.86 ± 0.01	-1.32 ± 1.62	19 ± 6
283.16	35.31	66,700	5.59×10^{-43}	193.35	6.84 ± 0.01	-1.32 ± 1.62	19 ± 6
278.16	35.95	69,500	2.98×10^{-43}	194.60	6.77 ± 0.01	-1.32 ± 1.62	16 ± 6
278.16	35.95	69,000	3.22×10^{-43}	194.45	6.76 ± 0.01	-1.32 ± 1.62	16 ± 6

*Rln K' = Rln (1/ K_{eq}) and K_{eq} = dissociation constant

The plot is shown in Figure 31. The slope of the curve is equal to the negative ΔH° for the association reaction. It gives a value of 1.35 ± 1.62 Kcal/ mole of monomer. The entropy change, ΔS° , for the same reaction was calculated by equation (41),

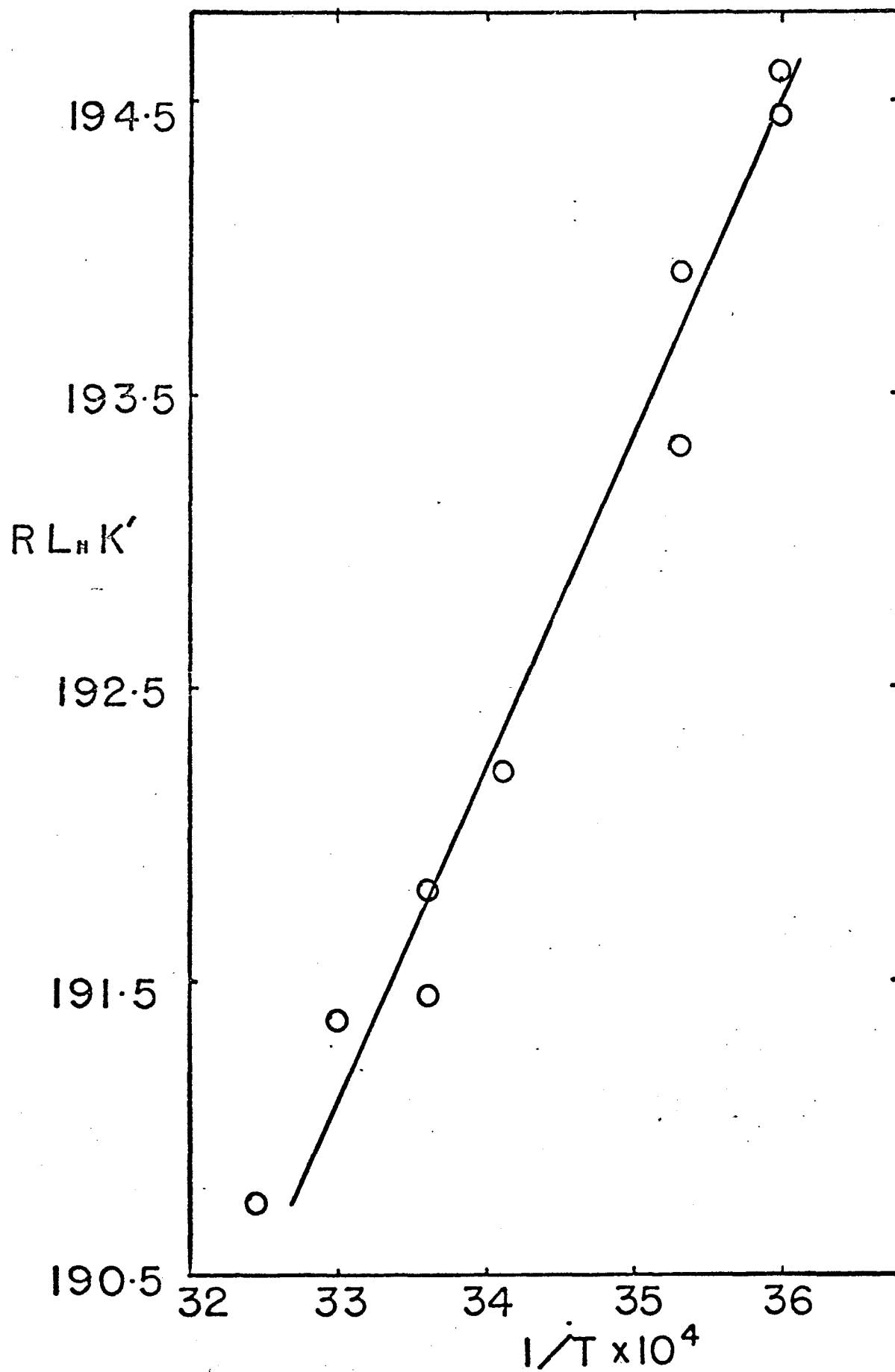
$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (41)$$

All these thermodynamic parameters for the association reaction of oxyhemerythrin are collected in Table XIX. The experimental error in enthalpy and entropy of the association reaction was also estimated according to Wiberg formulas (Wiberg, 1964), assuming that the fractional error of the association constant is 0.1.

Effect of Ionic Strength on the Association-Dissociation of Hemerythrin

The study of the effect of ionic strength on the association-dissociation equilibrium of oxyhemerythrin was carried out by varying the concentration of Tris-cac buffer and sodium sulfate in the reaction medium. This procedure was adopted

Figure 31. Enthalpy changes as a function of temperature.
Conditions: 30 $\mu\text{g/ml}$ oxyhemerythrin in 0.01 μ pH 7.00
Tris-cacodylate buffer.



because neither the components of the Tris-cac buffer nor sodium sulfate form coordination complexes with the iron of hemerythrin. These salts were therefore assumed to bind either not at all or only very poorly to hemerythrin.

In 0.01 μ Tris-cac pH 7.00 medium, the dissociation of oxyhemerythrin was a rapid reaction as evidenced by the presence of a single sharp leading boundary and an unresolved bimodal trailing boundary at different temperatures (Figure 32, curve a). When the ionic strength was increased above 0.1, the rate tended to decrease, particularly at low temperature. At 0.25 μ with Tris-cac buffer and at 10⁰, two well separated boundaries appeared both in the leading and trailing boundaries of the elution profile (Figure 32, curve b). From the sieve coefficients, the interacting species were identified as the octamer and monomer of hemerythrin.

When sodium sulfate was used to vary the ionic strength, elution profiles showing two separate boundaries were observed when the ionic strength of the solution was greater than 0.15 at 10⁰. The free energy changes as a function of ionic strength both with Tris-cac and with sodium sulfate are shown in Figure 33. Apparently, the displacement of the two curves cannot be entirely the result of ionic strength effects; the specific ion effect must also be operative.

Figure 32. Effect of ionic strength and temperature on the dissociation of oxyhemerythrin.

Curve AA: Hemerythrin concentration = 25 $\mu\text{g/ml}$; in 0.01 μ pH 7.00 Tris-cacodylate buffer and 25°.

Curve BB : Hemerythrin concentration = 25 $\mu\text{g/ml}$; in 0.25 μ pH 7.00 Tris-cacodylate buffer and 10°.

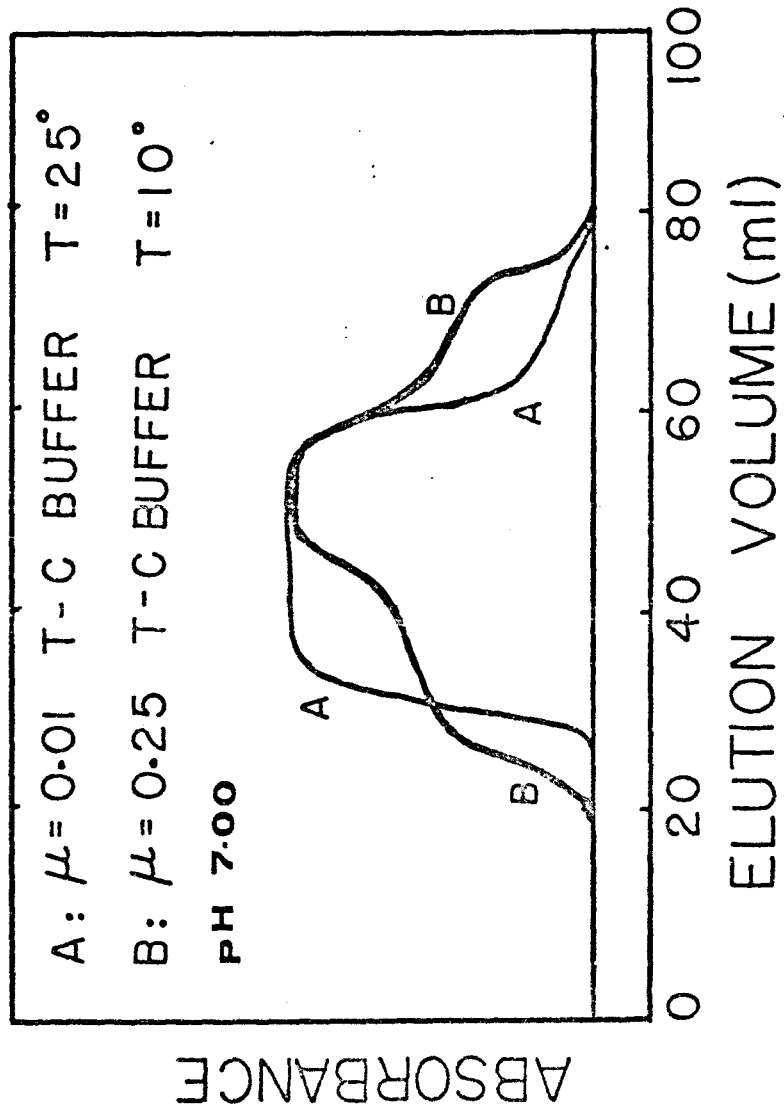
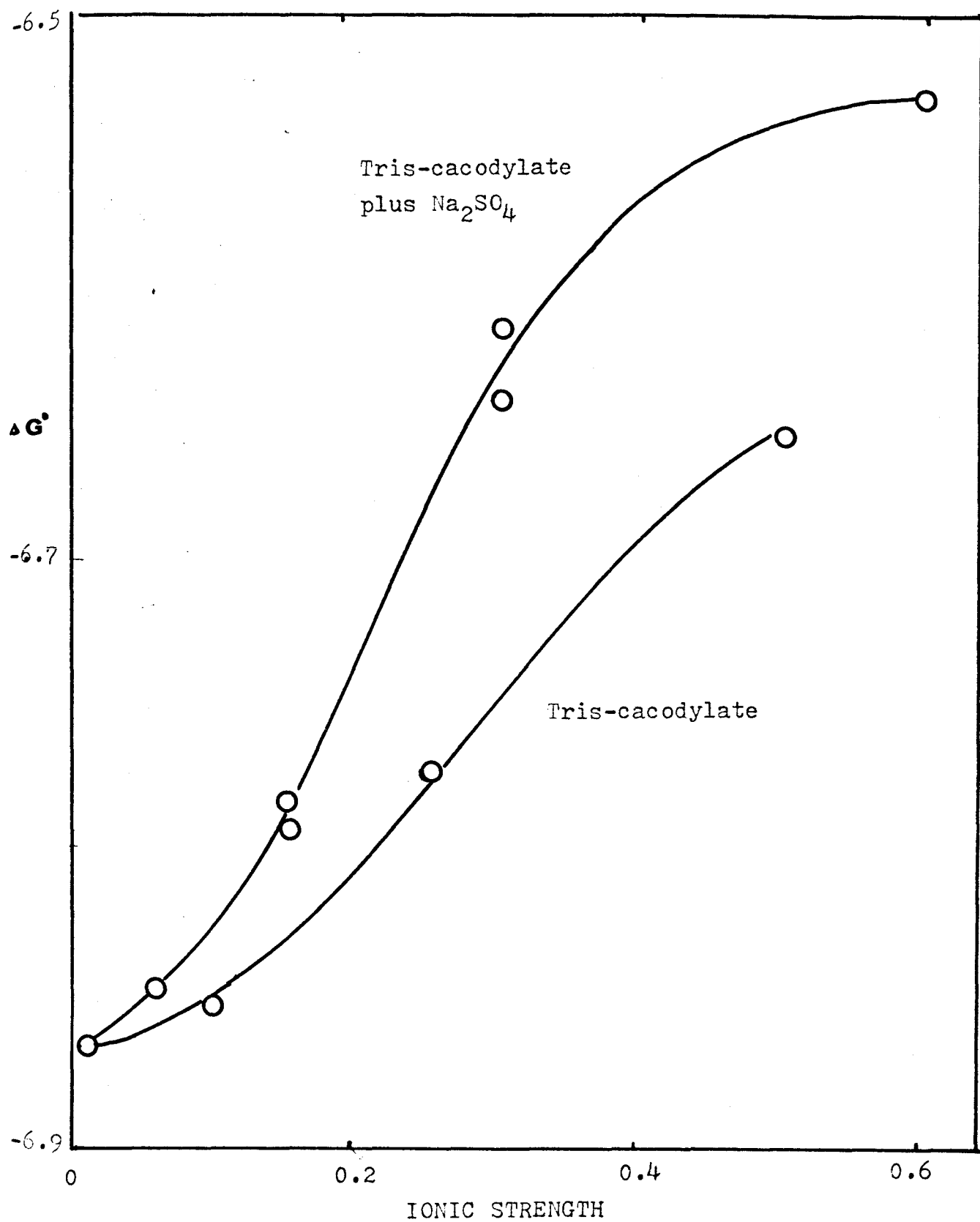


Figure 33. Dependence of the free energy of oxyhemerythrin dissociation on ionic strength. Open circles are data in sodium sulfate; filled circles are data in Tris-cacodylate. Conditions: 10 °C, 0.01 M Tris-cacodylate buffer, pH 7.00.



Somewhat different results were obtained at pH 6.00 and 10⁰. The integral and differential elution patterns are shown in Figures 34a and b. Although the extent of dissociation at pH 6.00 was expected to be greater than that at pH 7.00 at any given salt concentration, the K_{eq} versus ionic strength plot (Figure 35) surprisingly showed somewhat different patterns. The curve obtained at pH 6.00 was hyperbolic as in the Michaelis enzyme kinetic plot, while the curve at pH 7.00 was sigmodal.

When the data in Table XX were plotted as the $\ln K_{eq}$ versus the logarithm of the ion-pair activity, $\ln a_x$ (Robinson and Stokes, 1959) at these two pH, essentially a straight line was obtained as shown in Figure 36. The slope of the lines are 0.92 and 0.45 for pH 7.00 and pH 6.00, respectively. Wyman (1964) and Tanford (1969) have shown that the logarithm of equilibrium constant of a reaction can be expressed in terms of solution variables (Appendix 3). The total differential of $\ln K_{eq}$ at constant pH is indicated by Eq. (43),

$$d \ln K_{eq} = \left[\frac{\partial \ln K_{eq}}{\partial \ln a_x} \right]_{a_{H_2O}} d \ln a_x + \left[\frac{\partial \ln K_{eq}}{\partial \ln a_{H_2O}} \right]_{Q_x} d \ln a_{H_2O} \quad (43)$$

where x = sodium sulfate

Figure 34a. Integral elution profiles of oxyhemerythrin obtained at various ionic strength in sodium sulfate. Conditions: 10° , pH 6.00, 0.01 M Tris-cacodylate, protein concentration = 30 µg/ml.

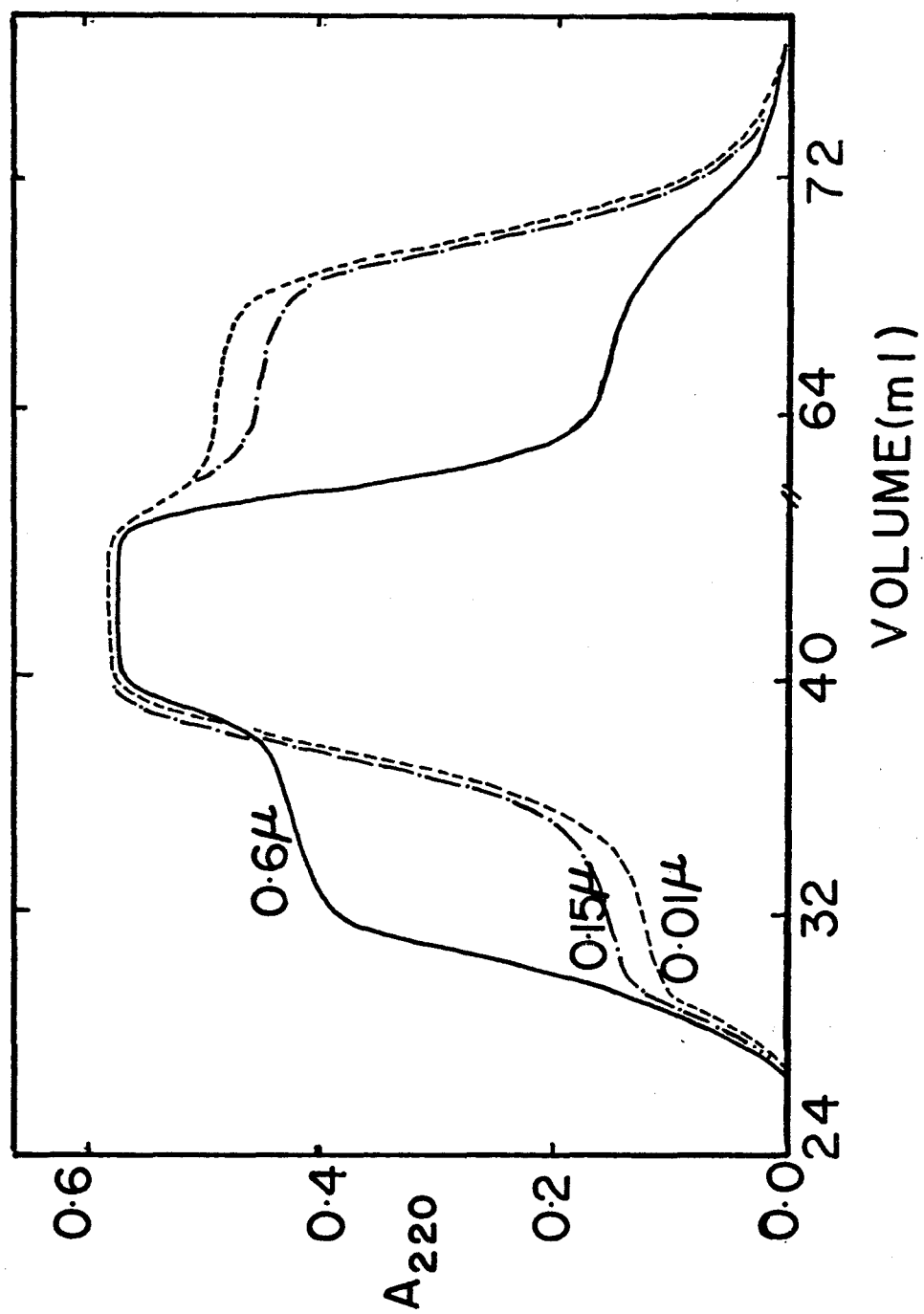


Figure 34b. Differential elution diagrams. Conditions were the same as those given in Figure 34a.

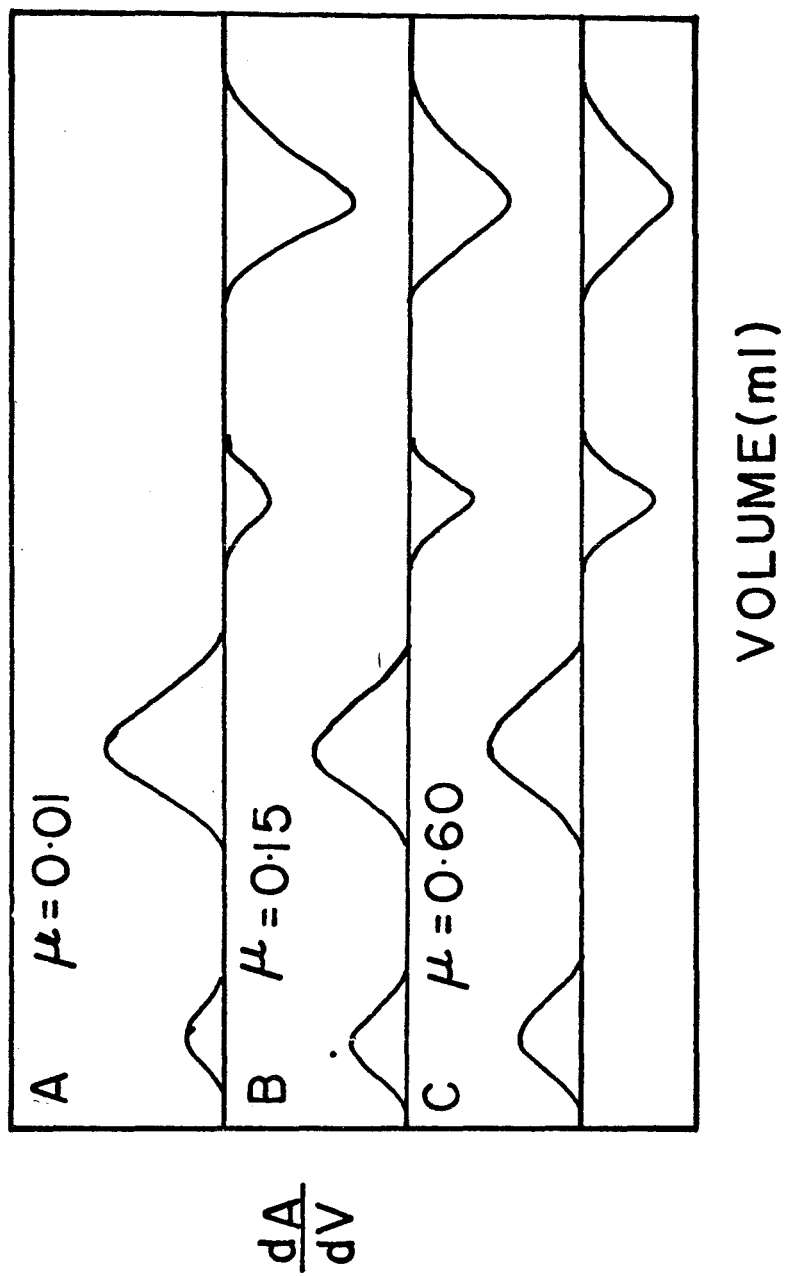


Figure 35. Dependence of the dissociation constant of oxyhemerythrin on ionic strength. Conditions: 10° , 0.01 M Tris-cacodylate plus sodium sulfate; the protein concentration was 30 $\mu\text{g/ml}$.

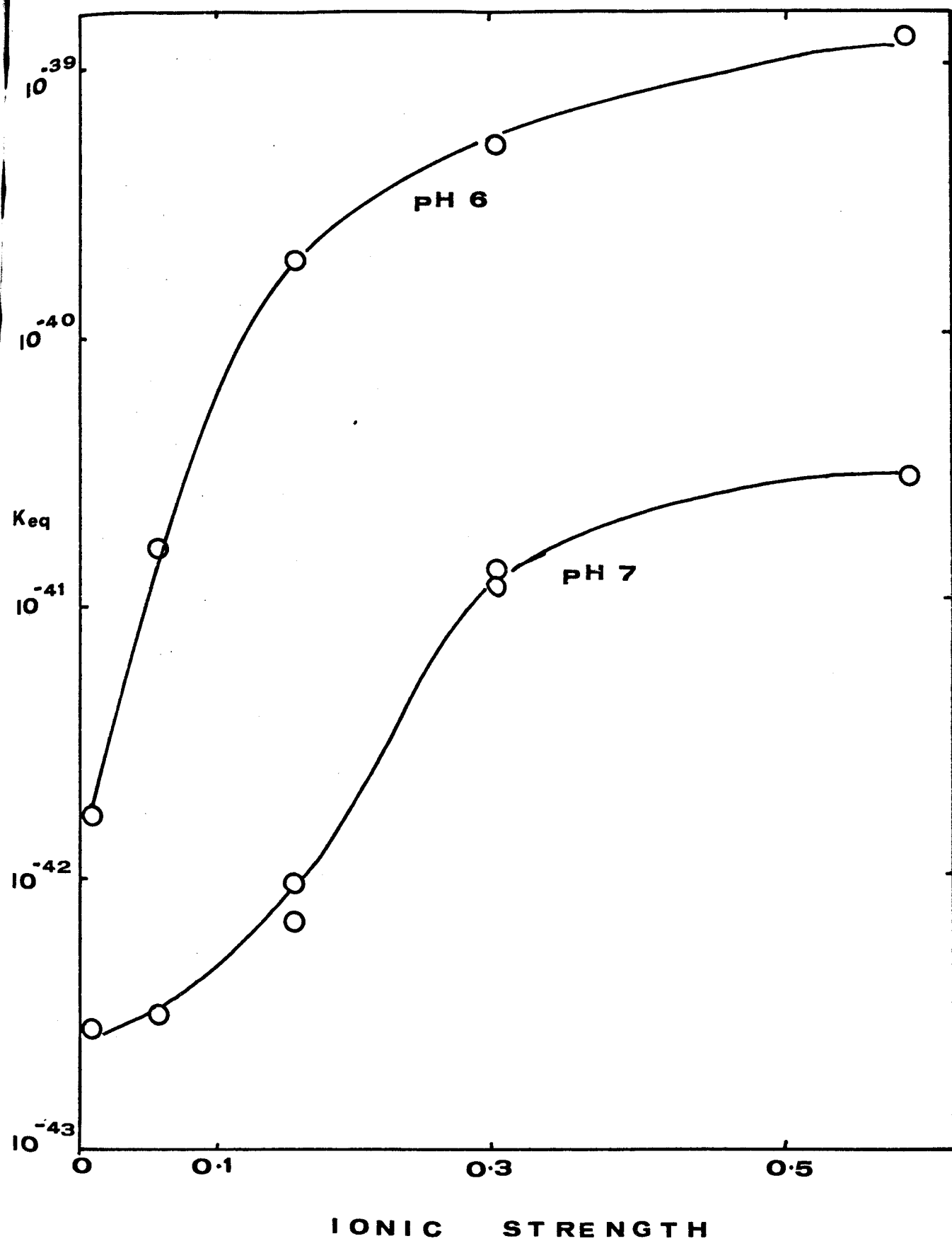
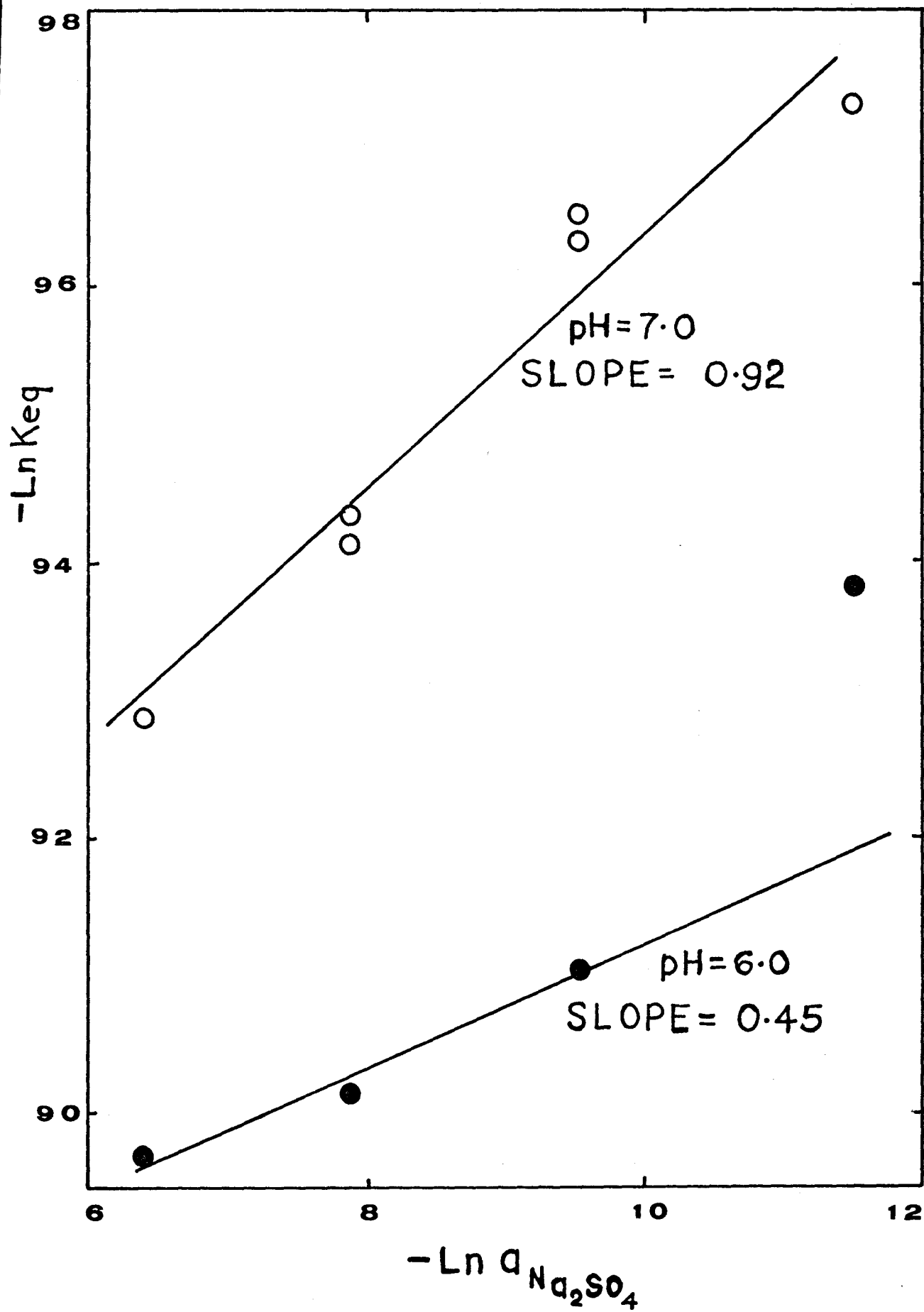


TABLE XX

Determination of the natural logarithm of the dissociation constant on the natural logarithm of sodium sulfate ion-pair activity at 10°C in 0.01M Tris-cacodylate buffer.

pH	Molarity	$\ln a_{\text{Na}_2\text{SO}_4}$	K_{eq}	$-\ln K_{\text{eq}}$	ΔG° (Kcal/mole) of monomer
7.00	0.00	-----	4.20×10^{-43}	97.593	6.864
7.00	0.02	-11.553	5.60×10^{-43}	97.306	6.844
7.00	0.05	- 9.564	1.43×10^{-42}	96.372	6.778
7.00	0.05	- 9.564	1.21×10^{-42}	96.540	6.789
7.00	0.10	- 7.906	1.07×10^{-41}	94.360	6.636
7.00	0.10	- 7.906	1.32×10^{-41}	94.145	6.622
7.00	0.20	- 6.417	4.73×10^{-41}	92.868	6.531
6.00	0.00	-----	2.18×10^{-42}	95.647	6.748
6.00	0.02	-11.553	1.74×10^{-41}	93.869	6.602
6.00	0.05	- 9.564	2.80×10^{-40}	91.091	6.406
6.00	0.10	- 7.906	7.01×10^{-40}	90.173	6.342
6.00	0.20	- 6.417	1.12×10^{-39}	89.706	6.309

Figure 36. Dependence of the natural logarithm of the dissociation constant on the natural logarithm of sodium sulfate ion-pair activity; 10^0 , 0.01 M Tris-cacodylate buffer; open circles: pH 7.00; filled circles : pH 6.00



Using Wyman's equation,

$$\left[\frac{d \ln K_{eq}}{d \ln a_j} \right]_{a_i \neq j} = \Delta \bar{v}_j \quad (44)$$

and rearranging Eq.(43) yields the expression

$$\left[\frac{d \ln K_{eq}}{d \ln a_x} \right] = \Delta \bar{v}_x + \Delta \bar{v}_{H_2O} \left[\frac{d \ln a_{H_2O}}{d \ln a_x} \right] \quad (45)$$

Tanford(1969) has discussed this equation and has shown that for multicomponent system, the quantity $(d \ln K_{eq} / d \ln a_x)$, is the difference between the preferential binding of solvent components, $\Delta \bar{v}_{pref}$, to the two end states of the reaction in question. The parameter, $\Delta \bar{v}_{pref}$, can be rewritten as

$$\Delta \bar{v}_{pref} = \Delta \bar{v}_x + \Delta \bar{v}_{H_2O} \cdot \left[\frac{d \ln a_{H_2O}}{d \ln a_x} \right] \quad (46)$$

Equation (46) [see Appendix 3] can be further simplified to Eq. (47),

$$\Delta \bar{v}_{pref} = \Delta \bar{v}_x - m_x / m_{H_2O} \cdot \Delta \bar{v}_{H_2O} \quad (47)$$

where m_{H_2O} and m_x represent the molalities of water and sodium sulfate, respectively. Thus, the slopes of the lines of $\ln K_{eq}$ against $\ln a_x$ plot (Figure 36) represent the parameter, $\Delta \bar{v}_{pref}$. For the dissociation of oxyhemerythrin in the neutral and acidic pH range, this quantity may be expressed as

$$\Delta \bar{v}_{pref} = (8 \bar{v}_x^1 - \bar{v}_x^8) - \left(\frac{m_x}{m_{H_2O}} \right) (8 \bar{v}_x^1 - \bar{v}_x^8) \quad (48)$$

where the superscripts 8 and 1 refer to octamer and monomer of hemerythrin. The values of $\Delta \bar{v}_{pref}$ were found to +0.92 and +0.45 for the reactions at pH 7.00 and 6.00, respectively.

CHAPTER IV

DISCUSSION

Many recent studies (Winzor et al, 1963) and 1964; Winzor, 1969; Ackers et al, 1965; Ackers, 1970; Kellett, 1967; Rao et al, 1972b) have demonstrated that gel chromatography is one of the most powerful and promising new approaches to the study of protein-protein interactions, especially in the lower range of protein concentrations (e.g. $<0.01\%$). The present study further demonstrates the versatility of this technique. More specifically, we believe that this technique is uniquely suitable to the study of interactions in hemerythrin over a wide range of experimental conditions of pH, ionic strength, temperature and protein concentration.

In this chapter, the following topics will be considered: First, the validity of using Sephadex-gel chromatography in general, and Sephadex G-75 in particular, in the study of subunit-subunit interactions in hemerythrin; Second, the nature of association-dissociation reaction of oxyhemerythrin, including the rate and the stoichiometry of the reaction . From the data on the concentration dependence of the weight average molecular weight and from the information on the stoichiometry in the pH region of 8.0 to 8.8, several reaction mechanisms will be tested for the

association-dissociation reaction in this particular pH range. Third, an evaluation of the contributions of various forces in maintaining the quaternary structure of hemerythrin will be made. The study of the effects of pH, ionic strength and temperature should provide the information required to make such an evaluation. Finally, we will consider the possible identities of some specific amino acid side chains participating in the subunit contacts of octameric hemerythrin. The data on the pK and the associated heat of ionization will be useful in identifying these specific residues. Additional chemical modification studies on this protein should provide confirmatory information concerning the specific residues at the subunit contacts.

Before the results obtained from Sephadex chromatographic experiment can be properly interpreted, I would like to comment on the validity of using Sephadex chromatography for the study of protein-protein interactions. First, it is necessary to establish that macromolecular interactions are not appreciably perturbed by the gel matrix so that the results can be interpreted in precise thermodynamic terms.

There are not many studies in the literature concerning the perturbation macromolecular interactions by gel matrix. Of the few, the best known are the studies of the polymerization of α -chymotrypsin (Winzor et al, 1963 and 1964; Gilbert, 1955) and the dissociation of carboxy -hemoglobin (Ackers et al, 1965).

marker proteins which are used to calibrate the column. The reason for this required condition is that the shape as well as the molecular weight determine the elution volume (V_e) of a protein on the column. In the present study, all the marker proteins used are globular proteins. The macromolecular properties of hemerythrin have been studied by Keresztes-Nagy (1962). He reported that native hemerythrin molecule was quite compact, with a low frictional ratio, f/f_0 of 1.13. Such a value is compatible with the molecular shapes of the marker proteins. The second condition is that the exclusion limit of the gel is appropriate for both the marker proteins and the macromolecule to be investigated. The octameric hemerythrin has a molecular weight of 107,000 and the oxyhemerythrin does not dissociate to any appreciable extent until its concentration is below 100 $\mu\text{g}/\text{ml}$. To assure that the weight average molecular weight of oxyhemerythrin remained in the linear partitioning range of the Sephadex G-75 gel matrix (<70,000 M.W.) and also that the sample solution had sufficient absorbance at the selected wavelength, I chose an optimal oxyhemerythrin concentration of 30 $\mu\text{g}/\text{ml}$ for these studies. However, oxyhemerythrin concentrations other than 30 $\mu\text{g}/\text{ml}$ were also used in several occasions, for example, in the studies of concentration dependence of dissociation and also in studies in the low pH region.

One may wonder why Sephadex G-100 or G-150 was not chosen

for studying the hemerythrin system. At first glance, Sephadex G-100 or G-150 appears to be a better choice than G-75 since the exclusion limit for the former gel is about 100,000 M.W. and that for the latter is about 150,000 M.W.. However, the maximum elution rate that can be attained on G-100 gel is limited to one half of that of a G-75 gel. Increased time on the column results in increased diffusion of the boundaries. In fact, the elution patterns obtained from G-100 are too diffuse for precise analysis. The choice of G-75 represents a compromise of flow rate and resolution range.

Nature of Association-Dissociation Reaction--Rate of Attainment of Equilibrium

The present study has demonstrated that the rate of the reaction of oxyhemerythrin could be altered by varying pH (Figures 12a and b and 13), temperature or ionic strength (Figure 32). When the pH was changed from 7.0 (and above) to less than 6.6, the observed elution profiles change from typical "fast" to "slow" patterns. The interacting species of hemerythrin present in the acidic pH region were completely separated during the transport experiment. They were identified as the octameric and the monomeric hemerythrin from the analysis of the individual elution volumes and sieve coefficients (Table VI).

The complete reversibility of the conversion of monomer to octamer was also established. The present study also demonstrated

that equilibration was rapid in all experiments above pH 6.6 in 0.01 μ Tris-cac buffer even at 5⁰. However, an increase in ionic strength reduced the rate of equilibration in the neutral pH region. At an ionic strength of 0.25 (Tris-cac) or 0.15 (sodium sulfate) at pH 7.00 and 10⁰, octameric and monomeric hemerythrin were completely separated on the column. This is consistent with a slow equilibration between the subspecies. An alternate explanation that protein was denatured by the higher ionic strength (from 0.1 to 0.6) is not in accord with the result of Rao (1969) who showed that the dissociation reaction to be reversible for met-hemerythrin up to 0.5 M sodium chloride.

The literature contains conflicting reports about the rate of association-dissociation reactions of hemerythrin complexes. Klapper et al (1966) studied the reaction of hemerythrin thiocyanate complex by ultracentrifugation, and reported that, since they were able to observe two boundaries and thus equilibration reactions were slow compared with the time of the experiment. Furthermore, the sedimentation coefficients did not change when the solution was diluted.

Rao et al (1972b) studied the same system under very similar conditions using gel filtration. They observed rapid equilibration and attributed the discrepancy between their results and the results of Klapper, et al to several factors

particularly to the difference in the pressure between their experiments and that from the sedimentation velocity experiments of Klapper et al. Rao and Kereszter-Nagy (1972b) also indicated that partial resolution of octameric and monomeric hemerythrin in the ultracentrifuge had been mistakenly identified as complete separation of the two components. In addition, there were other differences: First, the gel filtration experiments were done at an ionic strength of 0.1 while those of Klapper et al were closer to 0.2. The present investigation shows that the equilibration rates of hemerythrin subspecies are slow in solutions with ionic strength of 0.25 and at low temperatures. The species are completely separate only when the temperature is lower than 10° and $\mu \geq 0.25$ (Tris-cac). The interacting subspecies is only partially resolved at temperature higher than 15° even though they are in the solution with ionic strength as high as 0.5. Hence, the present study tends to agree with the interpretation given by Rao and Keresztes-Nagy about the rate of equilibration of hemerythrin species at room temperature and at low ionic strength ($\mu < 0.25$).

The Stoichiometry

Since I have described in detail the stoichiometry of the association-dissociation reaction of oxyhemerythrin in chapter III, this section will only discuss a few additional features. The association-dissociation of oxyhemerythrin in the acidic

pH has been demonstrated to be slow (Figures 12a and b) and completely reversible (Figure 17). In this case, the interacting species may be deduced from their sieve coefficients because the two boundaries representing the species were completely separated. The sieve coefficients of the fast and the slow moving components from the frontal experiments were found to be 0.040 ± 0.006 and 0.4835 ± 0.0153 , respectively. When these were compared with the σ_1 and σ_8 values of the pure monomeric (Table IV) and octameric (Table III) hemerythrin, the fast and the slow components were readily identified as the octamer and monomer. Therefore, the stoichiometry of the reaction is equal to 8 . The results obtained for the reaction in the neutral pH tended to confirm the earlier finding by ultracentrifugation (Keresztes-Nagy et al, 1965; Klapper et al, 1968) and gel filtration studies (Rao et al , 1972b) that the reaction corresponds to an octamer \rightleftharpoons monomer equilibrium. There are two lines of evidence from the present study in this regard. The first evidence is the direct measurement of the stoichiometry (n) by analyzing the V'_{\min} of the trailing boundary. The values of n are approximately 8 as indicated in Table XI. The second evidence is from the study of the reaction at high ionic strength and at low temperature (e.g. $\leq 10^\circ$). It has already been shown in the previous section that the rate of reaction at high ionic strength and at 10° is slow. Under these conditions the interacting species can be completely

separated and identified as octamer and monomer.

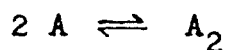
The elution profiles (Figures 14 and 20) for the reactions between pH 8.0 and 8.8 indicate a dimerization reaction rather than a polymerization process under a wide range of oxyhemerythrin concentrations. However, the stoichiometry of 2 does not necessarily mean that the reaction is a " simple monomer \rightleftharpoons dimer" equilibrium. That is , the stoichiometry does not provide the mechanistic picture of the reaction unless the number of species involved is actually known from some other measurement. Measurement of weight average molecular weight as a function of protein concentration confirms the presence of species intermediate between monomer and octamer but provides us no information about their number or nature.

Finally, the values of n (Table XI) for the reaction above pH 9.0 were equal to 3.7 ± 0.4 . Taken together with information on the experimentally determined weight average molecular weight, this suggests an octamer \rightleftharpoons dimer equilibrium (Tables VII--IX). Considering the variation of n (3.7 ± 0.4), one may question whether the reaction can be appropriately described as a simple octamer \rightleftharpoons dimer equilibrium.

Models of Association-Dissociation Reaction

The stoichiometry (n) of the reaction between pH 8.0 and 8.8 has already been shown to be equal to 2 (Figure 23).

According to the original Gilbert theory (1955), this can be interpreted as a simple dimerization involving only two species,



Depending on the measured values of \bar{M}_w , the dimerization could fall into any one of the following categories:

- (a) monomer \rightleftharpoons dimer
- (b) dimer \rightleftharpoons tetramer
- (c) tetramer \rightleftharpoons octamer

The wide range of \bar{M}_w measured between protein concentrations of 3.8 $\mu\text{g/ml}$ to 177 $\mu\text{g/ml}$ (Table XII) are compatible with neither of these three models above. For example, plot of $(\bar{M}_w - M_1) / (2M_1 - \bar{M}_w)^2$ versus C_0 (Figure 24) for monomer \rightleftharpoons dimer equilibrium shows an abrupt upward curvature at protein concentrations greater than 5 $\mu\text{g/ml}$. In the same manner, plots of $(\bar{M}_w - M_2) / (2M_2 - \bar{M}_w)^2$ and $(\bar{M}_w - 2M_4) / (2M_4 - \bar{M}_w)^2$ versus C_0 in Figures 25 and 26 suggest that both the dimer \rightleftharpoons tetramer and tetramer \rightleftharpoons octamer equilibria are by themselves insufficient to describe the association-dissociation reaction. These observations were therefore difficult to reconcile with the simple dimerization models involving

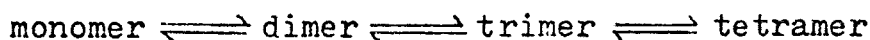
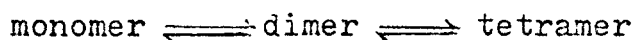
only two species.

Both a consecutive dimerization and continuous polymerization type dissociation reaction can give rise to a unimodal trailing boundary, even though both the original theories of Gilbert and Ackers et al did not consider these cases specifically. This conclusion is supported by the results of Chiancone et al (1968) on the dissociation of oxyhemoglobin and more recently by the work of Cassman and Schachman (1971) on the dissociation of glutamic dehydrogenase. Therefore, the data obtained on the dissociation of oxyhemerythrin between pH 8.0 and 8.8 is consistent with either a consecutive dimerization or a continuous polymerization model. The individual formation constants K_i' have been calculated on the basis of a consecutive dimerization process. The result indicated that the formation constant of octamer from the monomer is large (Table XIV). This leads to the prediction that octameric hemerythrin predominates at all but low protein concentrations (e.g. below 20 $\mu\text{g/ml}$). Figure 28 illustrates the relative amounts of various hemerythrin species at different protein concentrations. The tetramer is always present in minute concentration and the concentration of dimer is about 20 % of the total concentration. The bulk of the protein is thus distributed between dimer, monomer and octamer.

The consecutive dimerization model was tested in two ways.

First, the formation constants together with the values of α_1 and C_0 are used to calculate the theoretical weight average molecular weight (\overline{M}_w^C) through Eq. (33). The calculated \overline{M}_w^C are given in Table XV and plotted in Figure 27. These calculated weight average molecular weights fit the experimental curve reasonably well in the mid-range of protein concentrations. At very low and very high protein concentrations the fit is less good. This is probably due to the fact that the molecular weights obtained at very low or at high protein concentrations are subjected to larger variation on the Sephadex G-75 column. Second, the overall equilibrium constant for the consecutive dimerization reaction (Table XIV) was found to have about the same order of magnitude as the reactions in the neutral pH regions (Tables VII-IX). This is not surprising because the weight average molecular weights remained essentially unchanged for the reactions between pH 6.6 and 8.8 (Figure 11). More specifically, if the two end states of the association-dissociation reaction are the octamer and monomer, the thermodynamic parameter such as the equilibrium constant must be independent of the mechanism of the reaction. In that event, one should expect that the equilibrium constants would be the same.

In addition to the consecutive dimerization model, another possible model is the continuous polymerization model which may also fit the data. As mentioned previously, Cassman and Schachman (1971) studied the association-dissociation reaction of glutamic dehydrogenase system by sedimentation equilibrium. Their sophisticated statistical analyses of the results could not " distinguish " the consecutive dimerization and the continuous polymerization models,



Therefore, a continuous polymerization model may be valid for the oxyhemerythrin system in this pH region. The theoretical equation that describes this type of reaction has been derived by Steiner (1952). For hemerythrin, Steiner's equation becomes

$$\begin{aligned} C_o \bar{M}_w = & \alpha_1 C_o M_1 + 2K_2 (\alpha_1 C_o)^2 M_1 + \dots\dots\dots \\ & + 8K_2 K_3 \dots\dots\dots K_8 (\alpha_1 C_o)^8 M_1 \end{aligned} \quad (50)$$

The values of K_i may in principle be obtained by the graphical method as was explained in Chapter III.

However, it is emphasized that the determination of these successive association constants places heavy demands upon the experimental accuracy of the weight average molecular weights versus protein concentration data.

pH Dependence of the Association-Dissociation Equilibrium

The results given in Figure 11 indicates that the degree of dissociation of oxyhemerythrin is increased in both the acidic and the alkaline pH regions, while it is independent of the pH between pH 6.6 and 8.8. The effects of pH on the dissociation reaction could be examined in terms of electrostatic interactions between the charged groups. Thus, increased dissociation of oxyhemerythrin at low pH can

result from:

1. The elimination of favorable cationic-anionic interactions by protonation of the anionic groups;
2. Increasing unfavorable long range electrostatic repulsion between two macroions with a net positive charge;
3. Conformational changes accompanying protonation of the subunits causing the polymeric state to be energetically less favored.

It is known from other studies that the protonation of specific group can trigger gross conformational changes in protein molecule (Brandt and Hunt, 1967; Henn and Ackers, 1969). These gross conformational changes are usually accompanied by large enthalpy changes. The enthalpy changes we observe on the dissociation of hemerythrin tend to rule out the occurrence of such gross structural changes in the pH range studied. This is further supported by the circular dichroism spectral studies of hemerythrin-ligand complexes at various pH's by Garbett et al (1969). These authors reported that the CD spectra and thus the helical content (about 75%) did not change upon dissociation of octamer into monomer.

Nevertheless, the protonation to specific side chains can still promote subtle conformational change, which cannot be unambiguously detected either by calorimetry or by optical measurements. The enthalpies involved in such small conformational changes would probably be no greater than the enthalpies of ionization of the proton acceptors. If we assume no conformational changes are involved in the dissociation reaction of oxyhemerythrin, the pH dependence of the reaction can be analyzed in terms of ionizable groups fixed in space. When the logarithm of the dissociation constant is plotted versus $\log a_{H^+}$, Wyman (1964) has shown that the slope of the curve is equal to the number of protons involved in the reaction. According to Wyman's equation,

$$\left(\frac{\partial \ln K_{eq}}{\partial \ln a_{H^+}} \right) = \Delta \bar{\nu}_i = 8\bar{\nu}_H^i - \bar{\nu}_H^o \quad (51)$$

the slope is about +8 for the reaction at 25°. This means that each subunit of hemerythrin binds approximate 1.0 molecule of H^+ on dissociation of octamer to monomer. The simplest way of explaining this is to assume that one anionic and one cationic group are involved in the contact region for each monomer. In a similar fashion, the observed increased dissociation with decreasing proton concentration may be due to deprotonation of

a positively charged group such as $\epsilon\text{-NH}_3^+$ of lysine residue which might participate in the charge-charge interaction with the anionic group. However, other possibilities cannot be ruled out. For example, the effect of high pH may be to increase the net negative charge on the surface of the protein molecule creating long range electrostatic repulsion between subunits. Aromatic residues appear not to participate at the subunit contacts. This conclusion is supported by chemical modification studies by Fan and York (1971), as will be discussed shortly.

Specific Amino Acid Side Chains at the Subunit Contacts

The pH dependence of the dissociation of oxyhemerythrin (Figure 11) clearly suggests that at least one acidic and one basic amino acid residue is involved in the subunit contacts. The identities of these specific side chains may be determined if their pK values and apparent heats of ionization could be evaluated. Chemical modification of octameric hemerythrin and the resulting degree of dissociation may also provide information concerning the participation of specific side chains at the contact regions.

The values of pK_a and the apparent heat of ionization (Q') for the acidic residue has been obtained as described in chapter III. Table XVII shows the values of pK_a of the acidic side chain at various temperatures. At 25° , pK_a is equal to

5.08(and about 5.4 from ΔH° versus pH plot, see Figure 30) and Q' is equal to 2.6 ± 0.5 Kcal. These information may be useful in identifying the specific residue since the heats of ionization are generally characteristic of each type of amino acid residue(Wyman,1939). Combining the data on pK_a (5.08) and Q' (2.6 ± 0.5), the specific side chain may be deduced to be the γ -carboxyl group of aspartate or the δ -carboxyl group of glutamate according to the tables compiled by Edsall and Wyman (1958). On the basis of data on pK_a and Q' alone, the involvement of histidyl residue could be ruled out because its characteristic pK_a is about 5.5 to 7.0 and its apparent heat of ionization is close to 6 Kcal (Edsall and Wyman,1958). It is desirable to obtain additional evidence for the noninvolvement of a histidine residue. Chemical modification studies could serve this end. Fan and York (1969) did studied the possible role of histidine residues in hemerythrin. They undertook the chemical modification of histidine by DHT (5-Diazo-1-H-Tetrazole). Their results indicated that four out of the seven histidine side chains are bound to the two iron sites and the remaining three histidines seem to be free. Unfortunately, they did not carry out the parallel study on the degree of dissociation accompanying the modification of these histidine residues. The rather high pK_a for the carboxyl group may be the result of a shift in the intrinsic ionization caused by long range electrostatic interactions

among the macroions. Tanford (1961) has shown that long range electrostatic effects between the macroions become negligible only when the ionic strength of the solution is greater than 1. In the present case, the pH dependence of the dissociation equilibrium was carried out at ionic strength of 0.01. Thus, this might account for the unusually high pK_a for a carboxyl group. In order to test this suggestion, further experiments on the effects of ionic strength on the pK of this group should be performed.

The value of pK_a for the basic side chain is less certain than the acidic residue because the calculated equilibrium constants for the reaction above pH 9.0 were based on the dual assumptions that the stoichiometry was four and that only two interacting species were involved. Nevertheless, the approximate value of pK_a was found to be between 9.0 and 9.6. In the absence of data such as the heat of ionization and also the effects of chemical modification of specific residues, it is not possible to distinguish between tyrosine and lysine as the possible residues with these pK_a . Recently, York and Fan (1971) demonstrated that three out of the five tyrosines of both the native oxyhemerythrin and met-hemerythrin monomer were modified by TNM (Tetranitromethane). The remaining two tyrosine residues were reacted with TNM only when the protein had been acid denatured. This evidence strongly indicates that tyrosine is

not involved in the subunit contacts. Thus, it seems that lysine would be the most likely candidate with a pK_a of 9.0-9.6.

Recently, a residue with pK about 8.0 was reported by Langerman et al (1971) to be involved in the dissociation of hemerythrin on the basis of microcalorimetric studies. The authors interpreted this finding as evidence for the involvement of a cysteinyl residue. The pK obtained by Langerman et al was from the plot of ΔH^0 versus pH , assuming an octamer \rightleftharpoons monomer equilibrium in the whole range of pH from 5.6 to 9.2. I did not have sufficient data on ΔH^0 between pH 7.6 and 8.8 range so that I cannot comment on the possible involvement of cysteinyl residue at the subunit contacts. The role of the cysteinyl residue in hemerythrin has been the subject of studies by several workers (e. g. Egan, 1969; Cress, 1972). Nevertheless, our present study is in disagreement with the results of Langerman et al concerning the involvement of an acidic and a basic residue at the subunit contacts. Our evidence strongly suggest the participation of a carboxyl and a lysine residue, while Langerman et al were not able to demonstrate this.

Ionic Strength Effects

In this section, the relationship between hemerythrin-salt-water will be discussed. Since this is a three component system, according to thermodynamic principles, the chemical potential

of the components of a solution are interdependent, as specified by the Gibbs-Duhem equation. That is,

$$N_p d\mu_p + N_{H_2O} d\mu_{H_2O} + N_x d\mu_x = 0 \quad (52)$$

or
$$d\mu_p = - (N_{H_2O} / N_p) d\mu_{H_2O} - (N_x / N_p) d\mu_x \quad (53)$$

where N = mole fraction of a substance

μ = chemical potential

A change in the chemical potential of one component must obviously lead to a change in the chemical potential of at least one (and often both) of the others. Thus, the addition of a salt to a macromolecule-water system can change the μ_p in any of several ways:

It may exert nonspecific effects such as ionic strength effects on electrostatic interactions between macromolecules. The ions may interact with the macromolecule in two ways (von Hippel and Wong, 1964; von Hippel and Schleich, 1969). The first is the strong interaction between the ion and macromolecule resulting in the binding of ions to the macromolecule. The second is the so-called lyotropic effect (Hofmeister or specific ion effects) which is charge independent . Though the mechanism is unclear, ions such as SCN^- , I^- and Cl^- etc. are

particularly effective in dissolving proteins and also in dissociating non-covalently linked aggregates of proteins. The ordering of ions according to their effectiveness has resulted in the Hofmeister series (Hofmeister, 1888; von Hippel and Schleich, 1969; Jencks, 1969). The Hofmeister series may reflect salt induced changes in the structure of water. Finally, the salt may exert a combination of any of these effects.

A Brief Survey of the Effects of Some Specific Anions on Hemerythrin--Iron Coordination Ligands

Two groups of investigators using ultracentrifugation (Klotz and coworkers) and gel filtration (Keresztes-Nagy and Rao) techniques studied the effects of various specific anions such as azide, thiocyanate chloride etc. on the association-dissociation equilibrium of hemerythrin. Both groups found that under nearly identical conditions these anions readily coordinate with the iron in hemerythrin to give Hr-ligand complexes. Invariably, these Hr-ligand complexes dissociated to a larger extent than the native hemerythrin, namely, the oxyhemerythrin. Recently, Garbett et al (1971a) reinvestigated the problem of the binding of these specific anions to hemerythrin. They were able to demonstrate that the anions bind first to the non-iron sites and then to the iron atoms of hemerythrin. The rate of binding of anions to the non-iron sites was very fast and the excess of anions then form coordination complexes with

the iron. This means that it is difficult to tell which process (anion binding or ironcoordination) causes or controls the observed increase in the dissociability of hemerythrin-ligand complex. To resolve this problem, one needs to find anion which would bind well to the non-iron sites but which would form iron-coordination complexes at a relatively slow rate. Fortunately, chloride ion was found to fall into such a category. Fransioli (1969) and Rao (1969) studied the effect of sodium chloride on the dissociability of oxyhemerythrin. They observed from the indirect experiments that the increase in the dissociation could not be entirely due to the increase in total ionic strength and suggested that chloride ions imposed specific effect on hemerythrin (Table XXI). More recently, Rao further provided some direct evidence on the binding of chloride to the non-iron site by using labeled Cl^{36} and the chromatographic procedure of Hummel et al (1962). His results indicated that the binding of chloride ions to the non-iron sites caused an increase in dissociation and that the formation of the iron-Cl complex after chloride ions have bound to the anion sites had little effect on the dissociability of hemerythrin(Rao,1972a, p.75). Thus, we can conclude that it was the binding of the anion to non-iron sites which resulted in changes in the molecular properties of hemerythrin, that led to the increase in the rate of dissociation.

TABLE XXI

Effects of some specific anions on the dissociation equilibrium of oxyhemerythrin in 0.01 M pH 7.0 Tris-cacodylate at 25°.		
Protein concentration (g/l)	Conditions of dissociation experiment	Weight average Molecular weight
0.048	Hr-SCN	35,900*
0.048	Hr-SCN + 0.1 M KClO_4	41,200*
0.025	Aquo-Hr	54,300*
0.025	Aquo-Hr + 0.1M KClO_4	65,800*
0.025	Oxy-Hr	40,700#
0.025	Oxy-Hr + 0.1 M KClO_4	54,320#
0.025	Oxy-Hr + 0.1 M NaAc	35,500#
0.025	Oxy-Hr + 0.1 M NaBr	37,140#
0.100	Oxy-Hr (in 0.017 M buffer)	93,300#
0.090	Oxy-Hr (in 0.1 M buffer)	88,900#
0.100	Oxy-Hr (in 0.1 M buffer) + 0.1 M chloride	87,000#
0.098	Oxy-Hr (in 0.01 M buffer) + 0.5 M chloride	68,800#

* Compiled from Tables VI and VII(Rao, M.S. Thesis, 1969, Loyola University)

Compiled from Table XIII (Rao, Ph.D. Thesis, 1972)

Effects of Noncoordinating Anions

The same groups of researchers also studied the effects of some non-iron coordinating anions on hemerythrin. They found that perchlorate ion had a pronounced protective effect on the dissociation process and on the reactivity of the sulfhydryl groups of hemerythrin (Darnall et al 1968; Garbett et al , 1971a). On the basis of the spectral studies of hemerythrin solutions, Garbett et al suggested that perchlorate and chloride ions were bound to the same sites. Nitrate also inhibited dissociation but was only half as effective as perchlorate. In the same studies, Garbett et al further demonstrated that the sulfate did not bind to hemerythrin at all. Rao (1972a) also studied some effects of anions on the dissociation equilibrium of hemerythrin by Sephadex chromatography. He observed that several anions were quite effective in depolymerizing octameric hemerythrin (Table XXI). Rao attributed the increased dissociation of hemerythrin to the binding of these anions as well as to an increase in ionic strength of the protein solution. In order to resolve the problem of whether neutral salts promote dissociation by binding , by the ionic strength or by altering water structure etc., we studied the effect of sodium sulfate on hemerythrin. Sodium sulfate is an ideal choice because sulfate generally does not bind well to protein. We also used Tris- cacodylate as a means of regulation ionic strength, because Tris and cacodylate are also generally assumed not to bind to hemerythrin

and to other proteins.

The studies of ionic strength effect--by Tris-cac buffer and by sodium sulfate-- on the dissociation equilibrium of oxyhemerythrin were carried out at pH 7.00 and at 10^0 . The results are shown in Figure 34. The change in free energy as a function of ionic strength for the two sets of experiments were obtained. Obviously, displacement of the two curves cannot be explained entirely by the non-specific ionic strength effects; some specific ion effect must also be operative.

The effects of sodium sulfate were also investigated at pH 7.00 and 6.00 and at 10^0 . The results are expressed in terms of the dissociation constant versus ionic strength as shown in Figure 35. As expected, the degree of dissociation at pH 6.00 is greater than that at pH 7.00 at the same salt concentrations. The two curves, however, are different in shape. The curve obtained at pH 6.00 is hyperbolic while the plot at pH 7.00 shows sigmoidal behavior. The results shown in Figure 35 clearly indicate that sulfate ions are quite effective in dissociating hemerythrin into subunits at concentration greater than 0.05 M (or $\mu \geq 0.15$). A plot of the logarithm of the equilibrium constant ($\ln K_{eq}$) versus the logarithm of the ion-pair activity of sodium sulfate ($\ln a_x$) gives a nearly linear relationship. The slopes are about +0.92 and +0.45 for the dissociation at pH 7.00 and at 6.00, respectively. For multiple-

component systems, Tanford(1969) has shown that the quantity, $d\ln K_{eq} / d\ln a_x$, is the difference between the preferential binding of solvent components, $\Delta \bar{v}_{pref}$, to the two end states of the reaction in question. The parameter, $\Delta \bar{v}_{pref}$, can be rewritten as (see Appendix 3),

$$\Delta \bar{v}_{pref} = \Delta \bar{v}_x - m_x / m_{H_2O} \Delta \bar{v}_{H_2O} \quad (53)$$

where x = sodium sulfate

m = molality

In the case of hemerythrin, Eq.(53) becomes Eq.(54),

$$\Delta \bar{v}_{pref} = (8\bar{v}_x^1 - \bar{v}_x^8) - m_x / m_{H_2O} (8\bar{v}_{H_2O}^1 - \bar{v}_{H_2O}^8) \quad (54)$$

The slopes of the lines in Figure 36 represents the values of $\Delta \bar{v}_{pref}$ at pH 7.00 and 6.00. Let us consider the reaction at pH 7.00. The positive value of $\Delta \bar{v}_{pref}$ of approximately 0.92 may be interpreted in three ways according to Eq.(54). First, if there is no change in hydration during dissociation, i.e. the quantity $(8\bar{v}_{H_2O}^1 - \bar{v}_{H_2O}^8)$ is equal to zero, then the results suggests that about one more mole of salt is "bound" per mole of monomer than is "bound" per mole of native octamer. Second, if sodium sulfate does not bind to hemerythrin, namely, $(8\bar{v}_x^1 - \bar{v}_x^8)$ is equal to zero, then the monomeric hemerythrin contains about 60 fewer molecules of water of hydration than the octameric hemerythrin. The possibility that hydration

Decreases on dissociation is improbable in this instance. The process of dissociation has little or no effect on that portion of the protein molecule that was exposed to the solvent in the native octamer, so that the original sites for hydration are still present after dissociation, presumably without change in affinity for water. Furthermore, new sites for hydration are made available when the original contact areas between monomers become exposed to the solvent. A third possible interpretation is that both salt binding and hydration are increased when dissociation occurs. Since $\Delta \bar{v}_x$ and $\Delta \bar{v}_{H_2O}$ affect $\ln K_{eq}$ in opposing directions, larger values for $\Delta \bar{v}_x$ would be needed to account for the observed result when $\Delta \bar{v}_{H_2O}$ is positive than when $\Delta \bar{v}_{H_2O}$ is zero. Thus, if $\Delta \bar{v}_{H_2O} = 60$ moles water/ mole of monomer, $\Delta \bar{v}_x$ would have to be about 2 moles / mole of monomer to account for a value of $d \ln K_{eq} / d \ln a_x$ approximately equal to 0.92. In the absence of data from the direct binding study of sodium sulfate to hemerythrin, either the first interpretation or the third interpretation may be used to account for the value of $d \ln K_{eq} / d \ln a_x = 0.92$. At any rate, these two interpretation would not agree with the results of Garbett et al (1971a) who found that sulfate ions did not bind to hemerythrin. However, it should be emphasized that the conclusion drawn by Garbett et al was based on an examination of the spectra of hemerythrin in salt solutions and not a direct study of the binding of sulfate to hemerythrin.

We must reexamine the effect of sulfate on the association-dissociation of hemerythrin from the consideration of the salting out and salting in properties of this salt. The present study clearly indicates that the effect of sodium sulfate is the reverse of that expected from its salting in and salting out properties (Jencks, 1969). There is at least one other protein which shows the same behavior as hemerythrin in sodium sulfate solution. This is the dimerization of α -chymotrypsin at the acidic pH. The addition of sulfate to the protein solution tends to decrease the aggregation (Aune and Timasheff, 1971). This is in contrast to the prediction from the Hofmeister series. However, there are two proteins, hemoglobin and F-actin, whose dissociation behavior in salt solution follows closely the prediction described by the Hofmeister effect. Both hemoglobin and F-actin dissociate to a greater extent in solution containing perchlorate ion than in solution containing chloride, acetate or sulfate. The apparent contradictory behavior between hemerythrin and α -chymotrypsin on the one hand and F-actin and hemoglobin on the other may be resolved by a simple explanation. But before I explain this contradiction, I must discuss the salting in and salting out properties of these salts and try to relate their effects to the association-dissociation of protein molecules.

Salts which contain sulfate, phosphate, acetate or fluoride etc. tend to precipitate proteins and to protect them against denaturation and dissociation. On the other hand, salt which

contain thiocyanate, perchlorate, iodide, chloride etc. tend to dissolve, denature and dissociate proteins (Robinson and Jencks, 1965; von Hippel and Wong, 1964; Jencks, 1969). All of these changes in the physical state of protein involve a change in the degree of exposure of the protein to the solvent. In the case of association-dissociation reaction, areas on the surface of the protein monomer or subunit must become less available to the solvent upon aggregation to a polymer. Thus, salt solutions with which the surface areas can interact favorably will tend to favor the exposure of these areas to the solvent. Formally, the equilibrium for dissociation may be described according to equation (55),

$$K_{eq} = a_m / a_p = f_m C_m / f_p C_p \quad (55)$$

in which C, a and f refer to concentration, activity and activity coefficient, and the subscripts m and p refer to "monomeric" and "polymeric" protein, respectively. If the activity coefficients are defined as 1.0 for dilute solutions in water, an increase in the ratio of the concentration of "monomeric" to "polymeric" protein can occur if the activity coefficient of the polymeric protein is increased or, if the activity coefficient of the monomeric protein is decreased. Since the exterior of the protein is exposed to the solvent

in both the polymeric and monomeric states, dissociation must, therefore, involve a decrease in activity coefficient of the interior residues of the protein which become exposed to the solvent upon dissociation. That is, the rules for salting in and salting out just described have been established for the activity coefficients of the entire protein molecule (Scatchard and Kirkwood, 1932; Jencks, 1969). In other words, the interaction of solvent with the entire surface of the molecule is important. The process of association-dissociation has little or no effect on that portion of the protein molecule exposed to the solvent in the native molecule. When an association-dissociation reaction takes place, the interaction of solvent with the protein should be affected only in the region of intermolecular contact. Thus, if this region were to differ appreciably from the general surface of the protein molecule, a necessary thermodynamic consequence would be that solvent interactions would result favoring either the " monomer" or the "polymer" state. Indeed, Guidotti observed that the ability of a number of salts to dissociate the hemoglobin tetramer (1967) follows the Hofmeister series of ions. This is similar to that for the depolymerization of F-actin (Nagy and Jencks, 1966) and for the decreasing of the activity coefficient of the model compound of polyamide, acetyltetra glycine ethyl ester (Robinson and Jencks, 1965) . On the basis of the salt effect on hemoglobin dissociation , Guidotti agreed with Kawahara et al (1965) that

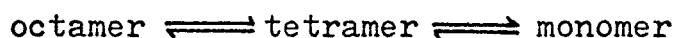
the surface of the contacting dimer ($\alpha_1\beta_2$ and $\alpha_2\beta_1$) contain groups, most likely peptide and amide groups, that are destabilized by specific ions and urea. These suggestions were confirmed by the X-ray diffraction studies on hemoglobin by Perutz et al (1968). Therefore, the opposing effect of sulfate on the dissociation of hemerythrin and hemoglobin reflects only differences in the nature of the groups at their respective subunit contacts. The data from the pH and ionic strength dependence suggest that ionic interaction plays an important role in maintenance of the octameric structure of hemerythrin. The thermodynamic data (Table XIX) is consistent with this view. It is apparent that the thermodynamic driving force is primarily entropic. The molecular picture of this increased entropy is not at all clear. Perhaps increased entropy is due to reorganizing of water molecules about the exposed groups or to the leaving of water from the domain of these groups on the formation of octamer, could cause increase in entropy. However, one must agree that the ultimate explanation of subunit interactions at the molecular level must await the determination of the three dimensional structure of hemerythrin by X-ray diffraction. Thus far, the subunit

contacts have been established only for horse hemoglobin with any degree of precision (Perutz et al, 1968).

Perspective

Numerous experiments can be suggested to extend the present investigation. We have demonstrated that the nature of the association-dissociation of oxyhemerythrin varied with the pH. From the properties of the elution profiles (Trailing boundaries) , we conclude that the predominant reaction of oxyhemerythrin at pH below 7.4 is an octamer \rightleftharpoons monomer equilibrium. Between pH 8.0 and 8.8, the reaction is changed to a dimerization process. Two reaction models have been proposed, the consecutive dimerization and the continuous polymerization, in order to account for the presence of the unimodal trailing boundary in the elution diagram. we have tested the consecutive dimerization model and found that it could fit the data reasonably well in the range of protein concentrations used.

It would therefore be necessary to test for the continuous polymerization model by further study of the concentration dependence of the dissociation. Moreover, the stoichiometry of the dissociation equilibrium above pH 9.0 is found to be close to 4. We assume that the reaction is a simple octamer \rightleftharpoons dimer equilibrium. However, other reaction models such as



might well be valid. It will be important to determine which reaction model (for pH 8.0-8.8 and for pH above 9.0) is correct so that the thermodynamic parameters can be measured. In other words, it is necessary to know the number of species present in the reaction mixture. Ackers (1968) has proposed a gel chromatographic procedure to enumerate the components in an interacting protein system. The theory behind this is involved and I will only describe it very briefly. The theory makes use of the fact that the individual sieve coefficient and weight average molecular sieve coefficient of a reaction mixture are functions of macromolecular concentration and of gel porosity. For an interacting system having k components, data must be obtained on at least $(2k - 1)$ columns of different porosities to obtain the weight fractions of all components in the system. The number of components can be completely determined when

$M \cdot N > k(M + N)$, where M = number of columns of different gel porosities and N = number of different reaction mixtures. Assuming there are four molecular species in the reaction mixture, it needs more than five gel columns with different porosities and also at least ten different protein concentrations in order to satisfy the requirement, $M \cdot N > k(M + N)$. Once the correct model is found for the reaction between pH 8.0 and 8.8 and also for that at pH above 9.0, one should study the temperature dependence of the reaction. Then, the data on the free energy , enthalpy, entropy changes and the pK values can be obtained.

We have indicated that carboxyl and lysine residues may be involved at the interface contacts between the subunits from the values of pK and the apparent heats of ionization. Some chemical modification studies on these groups and the accompanied change in the dissociation could provide confirmative evidence for their participation at the subunit contacts. Specific reagents have been developed which permit modification of carboxyl groups (Klippenstein, 1972) and free amino groups of proteins (Fan York, 1969)

Another experiment which should be done is to study the effect of organic solvent on the dissociation of hemerythrin so that the relative importance of hydrophobic forces in the subunit interactions could be further evaluated (we

have already suggested that hydrophobic force may make only minor contributions to the stabilization of quaternary structure of hemerythrin on the basis of changes in the entropy on dissociation at several temperatures (Table XIX). Churchilch et al (1963) observed that dioxane and sec-butanol promoted the dissociation of polymeric glutamic dehydrogenase lead to the suggestion that the forces holding the subunits together were largely hydrophobic in nature.

The most important experiment which should be done is to study whether various buffer components such as sodium sulfate bind to hemerythrin. The binding study can either be carried out with radioactive sulfate by the equilibrium dialysis method or more preferably by the gel filtration procedure developed by Hummel and Dreyer (1962). Metal ions binding may also be studied by comparing the effectiveness of sodium, potassium and ammonium sulfate in promoting dissociation. We observe that sodium sulfate greatly increases the dissociation of hemerythrin, a phenomenon which cannot be explained by the ionic strength effect alone (Figure 33). We prefer the interpretation that sulfate ions disrupt the salt-linkage and also that these ions bind stronger to monomers than to native protein,

although Garbett et al (1971a) concluded on the basis of spectral measurements that sulfate did not bind to hemerythrin.

Finally, it is interesting to find out what the quaternary structure or the spatial arrangement of the subunits of the native hemerythrin is. Since the native protein molecule consists of eight identical subunits, one very likely arrangement of the subunits seems to be the cubic structure. However, the kinetic data (e.g. Cress, 1972) on the reaction of p-chloromercuribenzoate with the cysteinyl residues of octameric hemerythrin always indicate that two out of the eight cysteinyl side chains react much faster than the rest of the residues. This could mean that not all the eight subunits in native hemerythrin are having the same "environment", as would be predicted by the cubic arrangement of the subunits. The problem of the spatial arrangement of the subunits in oligomer may be solved by the experiment in which octameric hemerythrin will be reacted with specific cross-linking reagent such as suberimide. The dissociation products will be analysed by gel electrophoresis. This method is originated by Davis and Stark (1970) and recently it is used quite successively by Carpenter and Harrington (1972) in elucidating the spatial arrangement of the protomers of leucine aminopeptidase system. In the case of octameric hemerythrin,

the cross-linked products may contain monomers, dimers, trimers, tetramers and so on. They can be separated by gel electrophoresis in pure forms. If this is the case, we can use these various species of hemerythrin to calibrate the Sephadex columns instead of using marker proteins to do the calibration. It should be very interesting to compare the two calibration curves for the same Sephadex column by these two sets of markers.

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APPENDIX 1

Derivation of the equation used for the calculation of equilibrium constant for simple polymerization

For the reaction, $n A \rightleftharpoons A_n$ (1)

the equilibrium constant for reaction (1) is given by equation (2),

$$K_n = \frac{C_n}{C_1^n} \quad (2)$$

and $C_o = C_1 + C_n$ or $C_n = C_o - C_1$ (3)

C_o represents the total weight concentration of protein.

C_1 represents the weight concentration of monomer.

C_n represents the weight concentration of n-mer.

C_i represents the weight concentration of i-mer.

The weight average molecular weight, \bar{M}_w , is defined by equation (4),

$$\bar{M}_w = \frac{\sum_i C_i M_i}{\sum_i C_i} = \frac{\sum_i C_i M_i}{C_o} \quad (4)$$

$$\begin{aligned} &= \frac{C_1}{C_o} M_1 + \frac{C_n}{C_o} M_n \\ &= \frac{C_1}{C_o} M_1 + \frac{(C_o - C_1)}{C_o} M_n \end{aligned} \quad (5)$$

Since $M_n = nM_1$ and when it is substituted into (5),

we obtain
$$\bar{M}_w = \frac{c_1}{c_o} M_1 - \frac{c_1}{c_o} (nM_1) + nM_1 \quad (6)$$

Solving equation (6) for c_1 ,

$$c_1 = \frac{(nM_1 - \bar{M}_w)c_o}{(n-1)M_1} \quad (7)$$

and
$$c_1^n = \left[\frac{(nM_1 - \bar{M}_w)}{(n-1)M_1} \right]^n c_o^n \quad (8)$$

Substituting equations (3) and (8) into equation (2), we get,

$$\begin{aligned} K_n &= \frac{(c_o - c_1) [(n-1)M_1]^n}{c_o^n (nM_1 - \bar{M}_w)^n} \\ &= \left[\frac{(n-1)M_1}{c_o} \right]^{n-1} \frac{(c_o - c_1) [(n-1)M_1]}{c_o (nM_1 - \bar{M}_w)^n} \\ &= \left[\frac{(n-1)M_1}{c_o} \right]^{n-1} \frac{(nM_1 - M_1)(c_o - c_1)}{c_o (nM_1 - \bar{M}_w)^n} \\ &= \left[\frac{(n-1)M_1}{c_o} \right]^{n-1} \left[\frac{n c_o M_1 - c_o M_1 - n c_1 M_1 + c_1 M_1}{c_o (nM_1 - \bar{M}_w)^n} \right] \\ &\quad \frac{1}{(nM_1 - \bar{M}_w)^n} \end{aligned}$$

$$= \left[\left(\frac{c_1}{c_o} M_1 - n \frac{c_1}{c_o} M_1 + n M_1 \right) - M_1 \right] \left[\frac{1}{(n M_1 - \bar{M}_w)^n} \right] \left[\frac{(n-1) M_1}{c_o} \right]^{n-1} \quad (9)$$

Substituting equation (6) into equation (9), we have

$$K_n = \frac{(\bar{M}_w - M_1)}{(n M_1 - \bar{M}_w)^n} \left[\frac{(n-1) M_1}{c_o} \right]^{n-1} \quad (10)$$

$$\text{or } K_n c_o^{n-1} = \frac{(\bar{M}_w - M_1)}{(n M_1 - \bar{M}_w)^n} \left[(n-1) M_1 \right]^{n-1} \quad (11)$$

$$\text{Plotting } \frac{(\bar{M}_w - M_1)}{(n M_1 - \bar{M}_w)^n} \left[(n-1) M_1 \right]^{n-1} \text{ versus } c_o^{n-1},$$

the slope of the curve is equal to K_n . For a dimerization reaction, monomer \rightleftharpoons dimer, equation (11) becomes

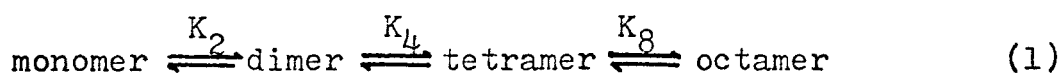
$$\frac{(\bar{M}_w - M_1) M_1}{(2 M_1 - \bar{M}_w)^2} = K_2 c_o \quad (12)$$

APPENDIX 2

Derivation of the equation used for the calculation of equilibrium constant for consecutive dimerization

i) Calculation of equilibrium constants

For the reaction,



we have,



$$K_2 = \frac{[\text{Hr}_2]}{[\text{Hr}_1]^2} \quad \text{or} \quad [\text{Hr}_2] = K_2 [\text{Hr}_1]^2 \quad (3)$$



$$K_4 = \frac{[\text{Hr}_4]}{[\text{Hr}_2]^2} \quad \text{or} \quad [\text{Hr}_4] = K_4 [\text{Hr}_2]^2 \quad (5)$$



$$K_8 = \frac{[\text{Hr}_8]}{[\text{Hr}_4]^2} \quad \text{or} \quad [\text{Hr}_8] = K_8 [\text{Hr}_4]^2 \quad (7)$$

where $[Hr_i]$ is the concentration of species i in gram per liter and K_i the individual formation constant of Hr_i . The overall equilibrium constant for reaction (1) is given by equation (8),

$$K_{eq} = \frac{[Hr_8]}{[Hr_1]^8} = K_8 K_4^2 K_2^4 \quad (8)$$

Since the total protein concentration, C_o , is the sum of all the species, we have

$$C_o = C_1 + C_2 + C_4 + C_8 \quad (9)$$

where C_i = concentration of i -species in g/l.

The concentration of C_i in equation (9) may be expressed in terms of C_1 through the relations (3), (5) and (7).

Hence,

$$C_2 = K_2(C_1)^2 \quad (10a)$$

$$C_4 = K_4(C_2)^2 = K_4[K_2(C_1)^2]^2 = K_4 K_2^2 C_1^4 \quad (10b)$$

$$C_8 = K_8(C_4)^2 = K_8[K_4 K_2^2(C_1)^4]^2 = K_8 K_4^2 K_2^2 C_1^8 \quad (10c)$$

Substituting equations 10a, b, c, into equation (9),

we obtain,

$$C_o = C_1 + K_2(C_1)^2 + K_4 K_2^2 C_1^4 + K_8 K_4^2 K_2^4 C_1^8 \quad (11)$$

$$= C_1 + K_2(C_1)^2 + K'_4 C_1^4 + K'_8 C_1^8$$

where $K'_4 = K_4 K_2^2$ (12a)

$$K'_8 = K_8 K_4^2 K_2^4 \quad (12b)$$

Let α_1 = weight fraction of monomer in the reaction mixture

$$= C_1 / C_o \quad (13)$$

Then, $C_1 = \alpha_1 C_o$ (14)

When equation (14) is substituted into equation (11),

we get,

$$C_o = \alpha_1 C_o + K_2(\alpha_1 C_o)^2 + K'_4(\alpha_1 C_o)^4 + K'_8(\alpha_1 C_o)^8$$

$$\text{or } C_o(1 - \alpha_1) = K_2(\alpha_1 C_o)^2 + K'_4(\alpha_1 C_o)^4 + K'_8(\alpha_1 C_o)^8 \quad (15)$$

The association constants, K_2 , K'_4 and K'_8 may be obtained by solving a set of three simultaneous equations (equation 15) if the variable α_1 at several protein concentrations can be determined. Steiner (1952) developed a graphical integration

method to obtain α_1 ,

$$\ln \alpha_1 = \int_0^{C_0} \left[\left(\frac{M_1}{\bar{M}_w} - 1 \right) / C \right] dC \quad (16)$$

When K_2 , K'_4 and K'_8 are determined, the association constants K_4 and K_8 are derived from the relationship illustrated by equations 12a and b. The overall equilibrium constant, K_{eq} , can then be calculated from K_2 , K_4 and K_8 through equation(8).

ii) Calculation of theoretical weight average molecular weight (\bar{M}_w^C).

$$\text{By definition, } \bar{M}_w = \frac{i \sum C_i M_i}{i \sum C_i} = \frac{i \sum C_i M_i}{C_0} \quad (17)$$

For the consecutive dimerization reaction of the type described by equation (1), equation (17) becomes

$$\bar{M}_w = \frac{C_1 M_1 + C_2 M_2 + C_4 M_4 + C_8 M_8}{C_0} \quad (18)$$

Expressing C_i in terms of α_1 and C_0 with the aid of equations (10) and (14), one can write equation (18) as

$$C_o \bar{M}_w = \alpha_1 C_o M_1 + K_2 (\alpha_1 C_o)^2 M_2 + K'_4 (\alpha_1 C_o)^4 M_4 + K'_8 (\alpha_1 C_o)^8 M_8 \quad (19)$$

$$= (\alpha_1 C_o) M_1 + 2K_2 (\alpha_1 C_o)^2 M_1 + 4K'_4 (\alpha_1 C_o)^4 M_1 + 8K'_8 (\alpha_1 C_o)^8 M_1$$

in which M_1 = molecular weight of monomeric hemerythrin
 = 13,500

The theoretical weight average molecular weight, \bar{M}_w^C , may be estimated at various protein concentrations (C_o) since values of α_1 can be evaluated from graphical integration and K_2 , K'_4 and K'_8 and also M_1 can be determined.

APPENDIX 3

Derivation of the total differential of the equilibrium constant in terms of solution variables(Tanford,1969)

Let M = macromolecule

X = ligand

W = water molecule

n_m = total number of moles of M

n_w = total number of moles of W

n_x = total number of moles of X

\bar{v}_x = average number of molecules of X bound to each macromolecule

\bar{v}_w = average number of molecules of W bound to each macromolecule

a_m = thermodynamic activity of M

a_x = thermodynamic activity of X

a_w = thermodynamic activity of W

Consider a solution containing a macromolecule,(M), a ligand (X), and water(W). We also consider the macromolecule as existing in the form of numerous species MW_iX_j and write K_{ij} for the equilibrium constant of the reaction



Equilibrium constants with $j = 0$ represent the constants for the formation of hydrated, but unliganded forms of M.

It follows from these definitions that

$$\bar{v}_w = \sum \sum i K_{ij} a_w^i a_x^j \quad (2)$$

$$\bar{v}_x = \sum \sum j K_{ij} a_w^i a_x^j \quad (3)$$

the double sum extending over all possible values of i and j . Equations (2) and (3) are written more conveniently as

$$\bar{v}_w = \left[\frac{\partial \ln \sum \sum K_{ij} a_w^i a_x^j}{\partial \ln a_w} \right]_{a_x} \quad (4)$$

$$\bar{v}_x = \left[\frac{\partial \ln \sum \sum K_{ij} a_w^i a_x^j}{\partial \ln a_x} \right]_{a_w} \quad (5)$$

a_x and a_w are not independent variables, and the right-hand sides of equations (4) and (5) therefore do not represent physically meaningful operations. If a_x is chosen as the independent activity, the experimentally measurable quantity at constant temperature and pressure, is

$$\frac{d \ln \sum \sum K_{ij} a_w^i a_x^j}{d \ln a_x} \quad (6)$$

which may be expressed in terms of the partial derivatives of equations (4) and (5) as

$$\frac{d \ln \sum \sum K_{ij} a_w^i a_x^j}{d \ln a_x} = \frac{\partial \ln \sum \sum K_{ij} a_w^i a_x^j}{\partial \ln a_x} + \frac{\partial \ln \sum \sum K_{ij} a_w^i a_x^j}{\partial \ln a_w} \cdot \frac{d \ln a_w}{d \ln a_x} \quad (7)$$

The relationship between $d \ln a_w$ and $d \ln a_x$ is given by the Gibbs-Duhem equation,

$$d \ln a_w = - \frac{n_x}{n_w} d \ln a_x - \frac{n_m}{n_w} d \ln a_m \quad (8)$$

Because the binding relations (4) and (5) , apply in effect to infinite dilution of M (i.e. $n_m = 0$), so that

$$d \ln a_w = - \frac{n_x}{n_w} d \ln a_x \quad (9)$$

Combining equations (4) to (9), we obtain the final result,

$$\frac{d \ln \sum \sum K_{ij} a_w^i a_x^j}{d \ln a_x} = \bar{v}_x - \frac{n_x}{n_w} \bar{v}_w \quad (10)$$

Consider an association-dissociation reaction,



$$K_{eq} = \frac{[M]^n}{[P]} \quad (11)$$

where P is the polymer in equilibrium with the monomers. Let K_{eq} be the equilibrium constant for this reaction in concentration units. It is assumed that equations (4) to (10) apply to each macromolecule separately, with equilibrium constants, $K_{ij,m}$ and $K_{ij,p}$, respectively.

Let $[M]$ be concentration of all M molecules;

$[M]_f$ be concentration of M molecules without any bound ligand or bound water;

$[P]$ be concentration of all P molecules;

$[P]_f$ be concentration of P molecules without bound X or bound W.

$$\text{Then, } [M] = [M]_f \sum \sum K_{ij,m} a_w^i a_x^j \quad (12)$$

$$[P] = [P]_f \sum \sum K_{ij,p} a_w^i a_x^j \quad (13)$$

Substituting equations (12) and (13) into equation (11),

$$K_{eq} = \frac{[M]_f^n \sum \sum K_{ij,m} a_w^i a_x^j}{[P]_f \sum \sum K_{ij,p} a_w^i a_x^j} \quad (14)$$

We wish to know the effect of the presence of ligand X on the K_{eq} . This is given by differentiating the logarithm of K_{eq} in equation (14),

$$\frac{d \ln K_{eq}}{d \ln a_x} = \frac{n d \ln \sum \sum K_{ij,m} a_w^i a_x^j}{d \ln a_x} - \frac{d \ln \sum \sum K_{ij,p} a_w^i a_x^j}{d \ln a_x} \quad (15)$$

Substituting equation (10) into equation (15) gives

$$\begin{aligned} \frac{d \ln K_{eq}}{d \ln a_x} &= n \bar{v}_{x,m} - \bar{v}_{x,p} - \frac{n_x}{n_w} (n \bar{v}_{w,m} - \bar{v}_{w,p}) \\ &= \Delta \bar{v}_x - \frac{n_x}{n_w} \Delta \bar{v}_w \end{aligned} \quad (16)$$

where $\Delta \bar{v}_x$ and $\Delta \bar{v}_w$ represent the changes in the numbers of bound ligand and water molecules that accompany reaction.

Sample calculation:

For the reaction, $Hr_8 \rightleftharpoons 8Hr_1$ in sodium sulfate solution, suppose $d \ln K_{eq} / d \ln a_x$ obtained from the slope of $\ln K_{eq}$ versus $\ln a_x$ is 0.92 and assume $\Delta \bar{v}_x = 0$ at $n_x = 0.10$ M and $n_w = 55.5$ M, equation (16) becomes,

$$\begin{aligned} 0.92 &= 0 - 0.10 / 55.5 \Delta \bar{v}_w \\ \text{or } \Delta \bar{v}_w &= (55.5)(0.92) / 0.10 \cong 500. \end{aligned}$$

For an octamer \rightleftharpoons monomer equilibrium,

$$\Delta \bar{v}'_w = \frac{\Delta \bar{v}_w}{8} = \frac{500}{8} \approx 62.$$

Namely, each monomer bound about 62 less water molecules than the octamer when the octamer dissociates into subunits.

Suppose both salt binding and hydration are increased when dissociation occurs and if $\Delta \bar{v}'_w = 62$ moles water per mole of monomer, then,

$$0.92 = \Delta \bar{v}_x - (0.10/55.5) (62)(8)$$

$$\bar{v}_x = 1.98 \approx 2$$

APPROVAL SHEET

The dissertation submitted by Kim Hock Tan has been read and approved by the undersigned member of the faculty of the Loyola University Stritch School of Medicine who served as director of the research program.

The final copies have also been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 14, 1973

Date

Allen Frankfurter

Signature of Advisor